ENHANCEMENT OF GERMINATION IN NATIVE WOODY SPECIES WITH PARTICULAR REFERENCE TO *ROSA CORYMBIFERA* 'LAXA'

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ABSTRACT

Commercial production of native tree and shrub species from seed can be unpredictable. *Rosa corymbifera* 'Laxa' is one such species of commercial importance as a rootstock, and is characteristic of the *Rosaceae* family. During the investigation, a standard commercial pretreatment was used to determine the unpredictable nature of this species with regard to germination. It was found that germination varied from as low as 2% in one year to a high of 63% in a subsequent year. The average germination was 26% over this period. This presents a very real dilemma to the grower with respect to meeting demand from highly unpredictable species. This dilemma is inherent in native tree production from seed.

Germination became high and predictable with the addition of a compost maker, Garotta. During the same five year period the lowest germination achieved was 75% and the highest 99%. The average germination was 89%. Germination was vastly increased in percentage terms as well as becoming reliable from year to year. The benefits to the grower in using this technique are potentially great in terms of time and resources. Not only is this pretreatment highly predictable, it is safe and easy to apply, unlike alternatives such as the use of concentrated sulphuric acid to burn off the seed coat.

During the pretreatment of *Rosa corymbifera* 'Laxa' the influence of microorganisms was assessed. Microbes were found within the pretreatment and their presence was established as being required to overcome the dormancy of the seeds. Low microbial numbers and activity were found in the commercial pretreatment, resulting in low germination. High microbial numbers and activity were found in the Garotta pretreatment, resulting in high and predictable germination. Total absence of microbes was found to result in zero germination.

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1. INTRODUCTION

1.1 Native tree seed

Seed germination is a limiting factor in commercial production of native trees. It is highly variable in terms of percentage and emergence. The commercial producer desires a highly predicable seed batch for both of these criteria. However, tree seed does not behave in such a manner and this causes problems in determining how much seed to harvest, treat and sow resulting in unnecessarily high expenditure. If large amounts of seed have to be handled due to low germination rates, this may cause other problems in terms of seed supply. Different sources other than locally grown seed may have to be sought to meet demand. This may result in imports of foreign seed.

Mature seeds of most native tree and shrub species require pretreatment prior to sowing. This is to overcome inherent dormancy characteristics, which may be exogenous or endogenous (Gordon & Rowe, 1982). Despite germination percentages being low, the seed is viable. The challenge is to pretreat seed to maximise germination.

1.2 Natural germination

With very few exceptions tree seed will not germinate immediately after maturation and dispersal from the mother plant. This is due to a mechanism known as dormancy which is common to almost all plant species, ensuring germination only occurs in the most conducive conditions possible. In the case of tree seed, if the seed germinated in the autumn it was produced, then the seedlings would most probably not survive the winter. Thus dormancy can be considered a natural defence to ensure survival of the species (Harper, 1977).

Harper (1957) stated that "some seeds are born dormant, some acquire dormancy and some have dormancy thrust upon them". He continued by categorising these into the states 'innate', 'induced' and 'enforced' dormancy respectively. Innate dormancy is when seeds leaving the parent plant are viable but do not germinate due to some condition of the embryo, endosperm or maternal structures. Induced dormancy is acquired from some after ripening experience and enforced dormancy is the inability to germinate due to environmental constraints.

These three categories described by Harper (1957) can be better described as only two distinct forms of dormancy. Gordon and Rowe (1982) describe dormancy actually manifesting itself in two distinct forms, endogenous (morphological or physiological) or exogenous (physical or mechanical). It is usually a combination of both which causes dormancy in tree seed.

a) Morphological dormancy is caused by immature embryos within the seed coat. The period of warmth already described allows embyros to develop to a state where they are receptive to the cold period (Schopmeyer, 1974).

b) Physiological dormancy requires exposure to cold conditions (1-5°C) of fully imbibed (moist) seed. The time required under such conditions to overcome physiological dormancy depends upon the depth of dormancy (severity) and is species specific. This form of dormancy release is not fully understood but would appear to affect the influence of growth regulators and mobilisation of storage products (Lewak & Rudnicki, 1977).

c) Mechanical dormancy is caused by a tough outer covering which is often thick and fibrous. However with prolonged soaking most are permeable to water (Schopmeyer, 1974). The best method for overcoming this is the natural process of warm conditions during autumn and spring. The process is speeded up artificially by controlling the environment ensuring warmth, moisture and aeration. Although chemicals such as acid are used in some species, they could prove unsuitable due to the permeable nature of such seeds.

d) Physical dormancy is removed gradually under natural conditions mainly by biological decay (Gordon & Rowe, 1982) and may take many years. The dormancy will be overcome once any portion of the seed coat is breached. In the natural environment the seed coat is breached, probably by decay. This decay would be caused by microorganisms in the natural environment, in the leaf litter for example where the seed normally falls. However, there is little, if no published work relating to this for tree species - a few writers mention decay very briefly, but with no citation or evidence (as pointed out by Bradbeer, 1988). The author has found the following to date;

- "It has been suggested that saprotrophs aid germination by breaking down the hard pericarps and testas..... During this time [stratification] microbial

weakening of the seed coat or the pericarp is also achieved. This is not done in large scale agriculture, though is important in forestry and horticulture." (Campbell, 1985).

- "The environment is important in softening hard coats that are impermeable to water. Microbial attack is thought to be important, as well as abrasion by soil particles." (Bewley and Black, 1994).

- "In nature, the seed coat may be broken down or punctured by mechanical abrasion, microbial attack, passage through the digestive tract of animals or exposure to alternating high and low temperatures which, by expanding and contracting the seed coat cause it to crack." (Mayer and Poljakoff-Mayber, 1975).

- "The mechanisms by which scarification is reputed to occur in nature owe rather more to observation and inference than to experimentation. However, one can find suggestions that natural scarification is brought about by such means as trampling by hoofed animals, uncompleted predation by animals (e.g. rodents, birds, insects), damage by fungi and soil micro-organisms, passage through an animal's digestive tract, and extreme changes in temperature. Although all of these mechanisms are feasible and there is little doubt that they occur, some of them probably affect only a minute proportion of the seed population of a species." (Bradbeer, 1988).

- "Early rains coincident with warm soil temperatures result in a partial breakdown of the seed coat due to biological or chemical action in the soil and the seed is rendered permeable at a time when soil temperatures favour rapid germination." (Trumble, 1937).

- "It would appear that rose achenes of many species may be put in the same category as those "Two-year seeds" (Crocker, 1948) which require a warm period in the soil to permit disintegration of the coat, followed by a period at low temperature to after-ripen the embryo." (Jackson and Blundell, 1963).

However, Pfeiffer (1934), from studies on Snowberry (*Symphoricarpos racemosus*) found that fungi or another agent capable of fibre decomposition was necessary for germination. She stated that seed would not germinate in the absence of fungi and concluded it was

entirely possible that fungi play a role in the disintegration of hard layers of other seeds or fruits, especially indehiscent forms, comparable with snowberry.

The cell walls of woody tissues, whether they be stems or seeds, are made up from three main components, cellulose, lignin and hemicellulose (Campbell, 1985). It is rare for a single species of microorganism to degrade these entire cell walls, and under natural conditions it is a succession of organisms which contribute to this process. Mineral content is crucial in microbial decay of woody tissue. Nitrogen is often quoted as being a rate limiting factor, though it is possible for phosphorus to also be in short supply (Campbell, 1985). In fact the crucial factor is not the total amount of mineral present, but the amount which is actually available to the micro-organisms.

The carbon:nitrogen ratio is often referred to when discussing rate limiting factors. The C:N ratio gives an indication of the amount of nitrogen and carbon available, and the higher this ratio the more limited the microbial growth. However this is complicated by the fact that this ratio is for total carbon and nitrogen, when in fact it is the amount which can be mobilised which is rate limiting. Therefore whether nitrogen is limiting in such systems depends not on the C:N ratio, but on the ratio of available nitrogen - which is much harder to determine (Campbell, 1985). It can be generally assumed that dead woody tissue such as that found in the seed coat of tree species has a high C:N ratio and decay would probably be limited by nitrogen.

If physical dormancy is overcome by microbes, then the conditions under which those microbes thrive would in theory cause maximum decay. Such conditions include warmth and moisture, and in the natural environment these conditions occur in the spring and summer. The same can be said for mechanical dormancy, as the seed coat is probably also breached by microbes. It is also during the warm period that some morphological dormancy is overcome i.e. seed is sometimes shed with immature embryos, and during the warm period natural 'after-ripening' occurs which completes the development (Rowley, 1956; Stewart & Semeniuk, 1965).

Thus, seed shed in the autumn would lie on the soil surface or within the leaf litter but little would occur to the seed due to cooling conditions. However, once the spring begins, microbial action will increase and the seed coat will decay. Once the seed coat has been breached then any physiological dormancy can be overcome. This is usually in the form of a hormonal block on germination caused by ABA (abscisic acid) (Roberts & Hooley,

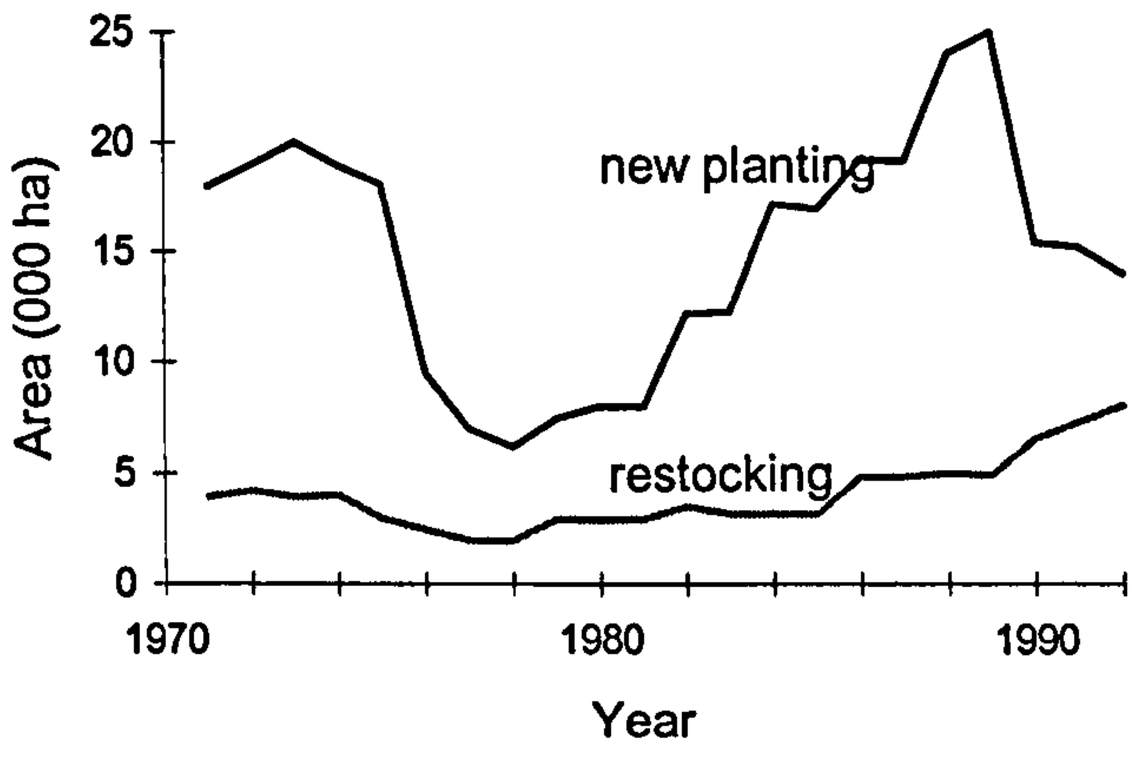
1988). Once the seed coat has been breached then water can enter and leave the seed freely and leach out the ABA - this is particularly effective in cooler conditions.

It can therefore be seen that natural germination comes about due to the seasonal changes, the warm months conducive to microbial growth and thus overcoming exogenous dormancy and the cold conditions overcoming the common endogenous dormancy.

1.3 Commercial native tree production

1.3.1 Trends in native tree production

Demand for native tree species, in particular broad-leaved species, has increased substantially over the last two decades. The actual area and therefore number of trees planted changes according to government budget legislation i.e. the level of grant aid for planting fluctuates from year to year. Figure 1.1 shows the area of woodland newly planted and restocked from 1971 to 1992 in the UK; original data taken from Forestry Commission annual reports.



taken from Aldhous, 1994.

Figure 1.1 Area used for new planting and restocking of private woodland, 1971-1992.

The graph shows a sustained increase in planting between 1978 and 1988. However, government aid was cut in 1972 and 1988, leading to a sharp drop in bare land afforestation (i.e. new planting). However, restocking was not adversely effected, and this increase is expected to continue for at least 50 years (Aldhous, 1994). The most recent decline in afforestation (1989-1992) should be reversed due to grants being increased for

planting, restocking and management of forests, with added incentives for planting farmland under the 'better land' supplement (Forestry Commission, 1995a).
The total amount of land forested in Great Britain either as new or as restocking by private landowners and the forestry commission amounted to an annual average of 33000 hectares over the 10 year period 1985 - 1995 (Forestry Commission, 1995b).

The increase in demand for native species is partly due to the reduction in use of agricultural land due to over production of food (as determined by the Common Agricultural Policy) and also partly due to an emphasis to return land to native deciduous forest (Forestry Commission, 1995a; Aldhous, 1994). It is also found that previous grubbing out of hedging (estimated at over 3220 km per annum since 1950 (ICI, 1988; Institute of Terrestrial Ecology, 1997) to increase individual field size has had detrimental effects such as wind erosion and depletion of species diversity of hedgerow wildlife (MAFF & RSPB, 1985). This has resulted in hedges now being replanted to address these problems.

1.3.2 The current and potential economics of the hardy nursery stock industry

The UK hardy nursery stock industry, which includes tree stock, was last valued at £252.9 million for the farm gate output in 1993 (MAFF, 1995). This was equally divided between field grown and container grown stock. Field grown trees accounted for 11.6% (£29m) of the total, 10% from field grown shrubs (£25m), 9.5% from field grown roses (£24m) with only 3.2% field grown herbaceous plants (£8m) (MAFF, 1995).

It can be assumed that the container grown stock would be similarly divided in terms of the ratio of each category. Therefore, following a similar trend this would result in another 12% (£30m) of output being container raised trees, 10% (£25m) container grown shrubs, 10% (£25m) roses and 3% (£7m) container grown herbaceous shrubs. Container grown stock would therefore account for two thirds (£173m) of the hardy nursery stock output.

Trees, roses and shrubs are all produced from seed, although some are produced by vegetative propagation. Thus it can be reasoned that if seed is the starting material for over two thirds of the hardy nursery stock, then it is a very valuable commodity to the grower. The importance of good quality seed at the outset of production cannot be over

emphasised, as quality seed will result in a quality crop of seedlings if handled correctly. Poor seed, however pretreated, will produce poor seedlings of low quality and yield.

It is therefore essential to start with the best quality seed possible as the quality of the resulting plants will be governed by it. Poor, cheaper seed is a false economy as poor quality plants will command a low price and be difficult to sell. The cost of seed is but a small fraction of the cost of producing a crop of trees, and Faulkner (1962) calculated that an increase of only half a percent in the value of the final crop justifies a 50% increase in the price of seed. Good quality seed and subsequent storage, treatment and germination will determine the quality and quantity of plants produced and thus their saleability in the market place.

In Britain between 80 and 120 million trees are used to restock or extend forests annually from British forestry nurseries alone (Aldhous, 1994). In addition to this, considerable numbers are exported to other EEC countries (Aldhous, 1994). Prior to the influence of man on the vegetation, about two thirds of Britain was covered by forest (circa 4000 BC) (Forestry Commission, 1995a). Records indicate that by the Domesday survey in the eleventh century, only 15% remained as woodland. The industrial revolution, ship building and the first world war (which put great demands on land for agriculture) meant that by 1918 the land area covered by deciduous woodland was reduced to 4%. In 1919 the Forestry Commission was established to manage, revive and protect forest land, and currently the land under woodland stands at approximately 10% (Forestry Commission, 1995b) .

Immediately following the first world war, afforestation of bare land dominated the planting activities across the country. However, more recently this trend has slowed and in the 1990/91 planting season 49% of planting was restocking. This reflected the ageing forests and the need to replace woodland which had been cut for commercial purposes. This percenatge is likely to increase as more woodland becomes mature and is cut and replaced (Aldhous, 1994).

As already mentioned, grants are available to accompany and encourage planting schemes. The ratio of broadleaves to conifers is likely to increase in the short term due to these grants being weighted in favour of broad-leaved species, and also due to increased public interest and awareness in woodland landscapes and wildlife habitats (Forestry

Commission, 1995; Aldhous, 1994). Higher aid is paid for broadleaf trees rather than conifers (Nix, 1995) and exact figures payable are quoted in the documentation from the Forestry Commission who operate the Woodland Grant Scheme (WGS) (Forestry Commission, 1995a). The main payment made is for creation of new woodland. During 1996, £700 per hectare was paid for conifer planting, compared with £1350 for broadleaf species. This latter figure dropped to £1050 for areas of new woodland planted over 10 hectares. Broad-leaved species can however be planted at half the rate of conifers (1100 plants compared to 2250 respectively). The obvious disadvantage is that broad-leaved trees cost on average 20-40% more to purchase than conifers (Oakover Nurseries, 1995), but the inflated grant and reduced stocking rate easily compensate for this and make broadleaves a much more attractive scheme.

Additional bonuses are available on top of the planting figure. A 'better land' supplement is payable to encourage woodland on arable or improved grassland sites (£600 per hectare), a £950 per hectare community woodland supplement to encourage woodland close to towns and cities for improved public recreation and a £600 per hectare location supplement for special initiative sites (but only for a limited period). Other grants include short rotation coppicing (£600 per hectare), annual management (£35 per hectare per annum) and restocking existing woodland (£325 for conifers and £525 for broadleaves, per hectare) (Forestry Commission, 1995a).

The community forest initiative also provided money for schemes involved in tree planting close to towns for recreational use, jointly funded by the Forestry Commission and Countryside commission (Anon, 1994). Advice on grant applications is available through the Forestry Commission by talking to a Forest Authority Woodland Officer (Forestry Commission, 1995a) and on design, species choice and silviculture techniques (Rodwell & Patterson, 1994).

With the current trend of increasing new woodland and replacing and reviving existing areas (section 1.3.1), native tree and shrub production remains an attractive business. The Woodland Grant Scheme funded through the Forestry Commission not only provides and maintains the physical resource of wood, but has further reaching consequences. Improved landscape for wildlife and recreation accompany the sustained economy of the rural environment, providing jobs and improved infrastructure in areas where agriculture once prevailed (Forestry Commission, 1995a).

As well as the woodland side of production, nursery output remains high, with more trees and shrubs being planted, especially in urban areas and along roadways. New novel outlets stimulate the trade, for example the use of thick, spiny species as natural barriers to potential trespassers. Market prospects are good for native trees and ornamental stock, and the knock on effect will result in a prospering seed industry.

Good quality seed from registered stands is at a premium and in limited quantity, and thus the ability to maximise its potential in terms of germination percentage is of paramount importance. Increasing the average percentage germination of all species could have beneficial consequences. It is the role of the seed scientist to attempt to achieve this, by studying techniques and applying the most suitable pretreatment to an individual species to enhance germination.

1.3.3 Native tree seed supply

The initiatives outlined in section 1.3.2 have increased the demand for native tree species and supply is met by the hardy nursery stock producers. Whether the stock required is for woodland, farmland or retail (Oakover Nurseries, 1995), all originates from seed. The most desirable seed source, and most expensive, is seed from nationally registered seed orchards or seed stands (Gosling & Aldhous, 1994; Forestart, 1995). The seed from such sources has known origin - the place in which a stand of native trees is growing; and provenance - the place in which any stand of trees is growing (whether native or not) (Lines, 1987). It is widely recognised that seed from stands of high inherent quality will result in high rates of germination and fast growing healthy plants producing good quality wood.

Thus seed from registered sources, whilst two to three times more expensive than unregistered stands of unknown quality, adds little to the overall cost of production (Gosling & Aldhous, 1994). The extra cost is amply covered by the increased growth and superior quality of the trees (Faulkner, 1992; Gill, 1983). Choice of seed origin is critical, and can result in a flourishing stand of trees or complete failure (Lines, 1987).

It would therefore be most desirable if seed from registered UK stands was always used in UK planting (Faulkner, 1992), however this is usually not the case due to limited and erratic availability. It is doubtful that such stands could provide enough material for the UK

market (Gordon (1995), personal communication), especially as many species do not produce a seed crop every year. It is therefore even more vital in such circumstances to enhance germination to a maximum, as increasing the percentage germination of a species in limited supply may overcome the finite quantity available. The problem of varying seed crops is a characterisitic of tree species, whereby poor seed set years are interspersed with 'mast' years - where a bumper crop of seed is produced (Harper, 1977). This characteristic of 'mast' years is not restricted to single trees, but manifests itself throughout a stand of trees (Harper, 1977).

Obviously as the seed is the starting material for trees and shrubs which may live for many decades, it is of utmost importance to ensure a good supply of quality seed and thus certification of source, provenance and registration is vital, especially to conform with EEC regulations (Gordon & Aldhous, 1992; Aldhous, 1994). This is becoming increasingly important as more seed is imported from such countries as Hungary, Chile and a variety of European countries (Forestart, 1995). Guidelines and recommendations for the main forest species in Britain are listed for new planting and restocking (Lines, 1987). Recommended origins and their sources are outlined by Gosling and Aldhous (1994).

1.3.4 Seed collection and processing

The first, and perhaps most critical stage in tree and shrub production is obtaining a good quality seed source. The majority of nurseries purchase their seed from reputable seed houses such as the Forestry Commission or Forestart. Seed from such sources will come with certification as well as information on germination. Seed obtained in this manner has undergone processing and would be ready to pretreat prior to spring sowing (Gosling & Aldhous, 1994). It is becoming more common to order seed in advance and have it delivered pretreated in the spring, avoiding the nursery performing the often difficult task of pretreatment (Forestart, 1995). Seed supplied in this manner will also be more reliable in terms of emergence and percentage germination as it has been handled expertly from harvest to delivery.

However, seed of broad-leaved and ornamental species is often collected locally from nursery stock or wild growing specimens (Gosling & Aldhous, 1994). This is also how the commercial seed houses obtain a lot of their seed, with the location of more rare species a closely guarded secret (personal observations).

Whether the seed is collected locally or purchased in, at some stage it has undergone handling, extraction (from berries for example) and storage. Processing requirements are closely related to morphological characters of the species and hence the combinations of cleaning and extraction are comprehensive. It would not be appropriate, for example, to process a *Corylus* nut in the same manner as a rose hip. Recommendations for the different species of seed can be found in the Forestry Commission Bulletins 59 and 83 (Gordon & Rowe, (1982) and Gordon, (1992) respectively).

Many of the native trees and shrubs have seed contained in a fruit or berry. These species represent the particular characteristics of the seed studied in this work, and hence will be briefly mentioned. Due to the nature of the fleshy fruit, these seeds need processing as soon as possible. The principle is to soften the fruits' wall in water before removing the seed from the pulp. Crushing or macerating the flesh can aid this process. Extraction methods vary between establishments and good techniques are often unique to individuals and as such are generally kept secret (Gordon & Rowe, 1982). Depulping involves extracting or separating the pulp from the seed and fruit remnants.

Cleaning follows the processing of the fruit, and can be performed either in the wet or dry state. This is usually achieved by sieving or winnowing. Few machines have been purpose built for tree seed due to the relatively small amounts involved and the great variability in seed size and structure (Gordon, 1992).

Drying prior to storage is a key factor in maintaining viability in a seed batch. This often occurs before cleaning as dry seed is usually easier to clean. A seed batch will be dried to a required moisture content based on weight loss of the wet seed. Without drying seed will continue to respire and viability will be lost. Problems of handling (such as caking) and disease may also arise with wet seed. Equally, seed dried too far will also suffer loss of viability (Gordon & Rowe, 1982).

1.3.5 Seed storage

The successful germination of tree seed requires great care and attention by the grower. If at any stage from time of collection to germination the seed batch is neglected, the consequences can be costly. Zero percentage germination can result in extreme cases (Wellington, personal communication, and authors experience). It is therefore important to understand the nature of the species before any treatment commences, with particular reference to its storage capabilities. Thus Roberts in 1973 coined the terms 'orthodox' to describe species whose seed could be dried to a low moisture content (5% or less fresh weight) without losing viability and 'recalcitrant' to describe those which are killed if dried a little below their fully hydrated condition (see definitions, appendix 1.1).

Recalcitrant seeds are particularly awkward to maintain from season to season and as such are often referred to as non storable seeds (Forestart, 1995). Seed lots are therefore processed quickly and sown as soon as possible. Storage even under optimal moisture conditions results in rapid loss in viability within a few weeks (longer in some species) (Roberts, 1973; Bewley & Black, 1994).

The most effective way to store such seeds is in fact to sow them immediately after harvest in the seedbed with adequate protection from vermin. Most of the recalcitrant species posses dormancy, and this will be broken by the natural conditions experienced over winter. If artificial storage is desired, then conditions similar to those in the seedbed should be chosen, e.g. moist peat. Such storage should occur in freely ventilated bags, and moisture should be carefully monitored to avoid drying out (Gordon & Rowe, 1982).

Orthodox broad-leaved species cover most families of trees and may exhibit a range of properties including hard seed coats, differences in size and shape, and a variety of dormancy levels from mildly dormant to deeply dormant. They have the great advantage over recalcitrant seed in that they can be stored over considerable lengths of time (months and often years) and be subjected to lengthy pretreatments to induce germination. The species covered by this work falls into this group.

To store orthodox seed for any period of time involves drying seed to approximately 10% moisture content and keeping at a constant temperature close to 0°C. Moisture contents nearer 5% and temperatures close to -18°C result in longer viability exceeding 5 years, but

it would be difficult to justify the added cost, especially when new, fresh seed should have become available during that time (Gordon & Rowe, 1982). To maintain moisture content, seed should be stored in plastic bags or bottles approximately 10-13 microns in thickness (400 - 500 gauge). Thinner material allows appreciable moisture exchange, and thicker prevents gaseous exchange - both of which are detrimental to seed storage (Bonner, 1978).

The research on commercial coniferous forest tree species is extensive with regard to optimum storage conditions, although broadleaved species have been somewhat neglected (Gordon, 1992). However, some data are available and recommendations can be found in the appendices of the Forestry Commission Bulletin number 59 (Gordon & Rowe, 1982).

1.3.6 Seed sowing and after care

The hardy nursery stock producer has a difficult decision regarding sowing density of a seed batch. If germination rates can vary within a species, and between species, then an educated guess is required. Seed with a germination rate of 10% would not be spread at the same rate as one with 85% - but how does one know? Obviously one way to overcome this problem is to perform a germination test prior to sowing - however with many species this can take weeks or months and is therefore not practicable (ISTA, 1993). An estimation of rates is usually made by the nurserymen based on experience. Some commercial seed houses will provide germination data and also, perhaps of more concern to the grower, field factors (Forestart, 1995). This is an estimate of the percentage survival after field sowing and can be calculated for any species providing data are available (Gordon & Rowe, 1982; Aldhous, 1994). It would therefore be of great value if the overall percentage germination could be enhanced to increase the density of sowing and reduce area used. This general principle has been the goal of nurserymen since commercial production of woody species began over 50 years ago.

The preparation of the area chosen for seedbeds follows a routine cultivation regime, with ploughing and cultivation of the land in the autumn. The soil is then sterilised over winter using a slow release sterilant such as Dazomet and covered with plastic. As soon as the ground is accessible in the spring the plastic is removed and the ground fertilised. Prior to sowing, the soil is consolidated and tilthed.

The pretreated seed will then be sown typically between mid March (in the south) and mid May (in Scotland). Sowing densities will have been calculated using the information available; guidelines can be found in the Forestry Commission booklet on nursery practice (Mason, 1994). Seed is either broadcast or machine drilled, in the latter seed must be dried to allow easy flow. Lime free grit is applied to the seed bed which actually promotes faster germination and higher yields (Mason, 1994). This is due to the protection it provides the seed by preventing drying out and also maintaining a higher average seed bed temperature. The latter is due to appropriate colour and size grit absorbing and retaining more heat (Mason, 1994). Protection from birds is provided by netting, and where rodents may be a problem, traps are set. Seed eating birds represent the most serious hazard from a predation point of view (Tee & Petty, 1973).

Aftercare on the nursery site involves weed and pest control, sun and frost protection, the application of top dressing fertilisers and irrigation (Aldhous, 1994). At the end of the growing season many species need to be transplanted to allow continued development. A pre-prepared seedling bed is required for this. Recommended areas vary depending upon age and species, but 75 to 200 square centimetres per plant is usually allowed (Mason, 1994). This translates into only 44 to 178 plants per square metre after correct spacing. If production requires tens of thousands of individuals, large areas of land are required. To ensure only high quality plants are continued into the transplant bed, grading is required at the transplant stage (Mason, 1994). This usually divides the plants into two height categories and discards poorer specimens. Graded plants are planted by hand or machine, but in both cases it is important to plant in straight rows for further husbandry actions. At the transplant stage undercutting may also be required to check root growth and ease planting.

All of these processes put heavy demand on space and labour, as well as on the amounts of water and consumables used on a unit area basis. Any reduction in land area required would reduce labour and consumables and hence reduce costs. Field grown Hardy Nursery Stock was estimated to cover 8272 hectares in (MAFF, 1995). This shows that the industry invests a lot of land area and subsequently time and effort into producing this stock and any saving in land would represent a relatively large saving in expenditure. Increasing the reliability of seed germination of tree seed would help by allowing accurate forecasting of sowing rates, thus achieving optimised land use.

1.4 Native tree seed germination

1.4.1 Inherent variability affecting production

Traditional processing of any given species begins with collection and cleaning of the seed to remove any fruit (or berry) and associated debris (see section 1.3.4), followed by treatment prior to sowing. Such pretreatment takes the form of subjecting the seed to ambient temperatures in moist media during the autumn, winter and spring in open frames. This is sometimes referred to as stratification and has now become a common term to describe all forms of moist conditioning of seed (U.S. Department of Agriculture, 1974) see appendix 1.1 for full definition.

Once the seed begins to show signs of chitting (i.e. germinating - see appendix 1.1 for full definition) it is sown in pre-prepared sterile (to kill weed seed) seed beds and protected from frost (using a layer of gravel (Mason, 1994; D. Fordham, personal communication)) and pests (by netting) and watered when appropriate. The period of pretreatment, media used and temperature regimes employed are the key factors involved in overcoming dormancy in any particular species (Gordon & Rowe, 1982). If seed fails to germinate in otherwise conducive conditions, i.e. it is dormant (Bradbeer, 1988), then it is the pretreatment that is the key to germination. It is dormancy which causes the most problems to a hardy nursery stock producer; not only because it may manifest itself in several ways including low percentage germination, sporadic emergence and differences in embryo maturity resulting in a poor yield of useable seedlings; but also because such a plethora of factors can cause and influence dormancy that the task of predicting germination rates is rendered almost impossible. Germination has been shown to vary from location to location (Wang, 1980) and season to season in seed collected from the same tree, probably due to prevailing weather conditions during fruit set, in particular drought (Rolston, 1978; Gassner, 1938).

In 1980 germination in Britain was 25% lower than expected due to the long cool dry spell that followed sowing (Gordon & Rowe, 1982). To calculate a field factor, records should be kept of weather, seed predation, drought, frost, drying winds, browsing and seedling loss on a particular nursery for each species. The figure for an individual seed lot cannot be known in advance, but can be predicted (Aldhous, 1994).

Due to the variation encountered with tree seed germination from season to season within a species (Rolston, 1978), researchers have devoted much time and effort to increasing percentage germination and speed of emergence. It is perhaps not surprising that tree seed germination has been the subject of much research and literature over the last decades. All aspects have been covered from biochemical studies, with particular reference to hormones (Jackson (1968) for a review with respect to *Rosa*), to after ripening and germination (reviewed by Amen (1968) with early work by Tincker and Wisley (1935) on rose seeds).

However, it soon became apparent to the author, that although much had been published with reference to tree seed germination (very little of which is recent), the majority covered the hormonal aspects involved, with no definitive works answering both how and why a seed may be dormant and then how this is overcome. Many papers found varying levels of endogenous hormones during pretreatment of seed, such as a decline in abscisic acid in *Rosa* (Tillberg, 1983; Yambe *et al*, 1992a), *Crataegus* (Qrünfleh, 1991) and *Acer* (Webb & Wareing, 1972; Pinfield & Davies, 1978) to cite just a few. However, evidence is circumstantial, with a rise or fall of a particular hormone coinciding with alleviation of dormancy or increase in germination.

Seed of many species fail to germinate due to the restriction of water uptake caused by a hard seed coat. This is not the case for all species, and care must be taken when referring to particular examples. Rose achenes imbibe rapidly as found by Tincker and Wisley (1935), Semeniuk *et al* (1963), Svejda and Poapst (1972) and the author. Investigations by Tincker and Wisley (1935) included studies using acid scarification and mechanical breaking of the seed coat. Subsequent testing showed no germination. The conclusion drawn was that germination was not delayed by the inability to take up sufficient water.

Jackson and Blundell (1963) isolated an inhibitor from *Rosa arvensis* which prevented germination when reapplied to naked embryos. This inhibitor, ABA, was located in the pericarp of the seed (Jackson, 1968) which explains why the excised embryos germinated when removed from the coat. Achenes of *Rosa hybrida* L. fail to germinate under suitable conditions whereas excised embryos germinate readily. This germination of the embryos is however inhibited if the pericarps are re-introduced to the incubation media (Yambe *et al*, 1992a). These workers showed that aerated washing of achenes with water resulted in leaching of the inhibitor causing dormancy. The addition of charcoal to the water increased final percentage germination, and when analysed was found to contain ABA. Yambe *et al*

(1992a) also showed this ABA leaching to be more effective at 5°C than 25°C. Germination increased from 25% at 25°C to 50% at 5°C.

The finding by Yambe and co-workers (1992a) that 5°C was more effective than 25°C in leaching ABA from *Rosa hybrida* L. is consistent with the physiological dormancy which is overcome in most species by the stratification process. Colder conditions are used to enhance leaching and breakdown of inhibitors, although it is also possible in this case that the colder temperature of the water may aid leaching by having a higher oxygen level (Forestart, 1995) than the warmer water. This could help bind and remove ABA more effectively.

It should be stressed that although the hard seed coat may prevent, or at least retard, water uptake, this is not the only factor determined by the seed coat. In the case of many woody species, the seed coat acts as a physical constraint to germination (Amen, 1968). However, invariably it is more than one factor determining dormancy, often a combination of a hard seed coat and consequently hormonal control (Rolston, 1978). Whilst water may be able to penetrate the coat, continuous circulation in and out from the embryo does not occur, and thus leaching of any inhibitors is unlikely.

These factors help explain why the usual warm/cold pretreatment for hard coated seed would overcome dormancy. Warm temperatures facilitate breakdown of the seed coat, whilst the subsequent cold period allows leaching and breakdown of any hormonal inhibitor. This concept can be extended to explain the need for a natural 18 month treatment to germinate hard coated species, including rose seed in Holland (Besten, (1995) personal communication). Seed harvested in the autumn of one year does not have suitable time before winter to allow the breakdown of the seed coat, and is thus still dormant following the spring and will not germinate. The warmer conditions of the spring and summer will allow decay of the pericarp in the natural environment (Jackson & Blundell, 1968). However a cold spell is still required to induce germination (i.e. the second winter following harvest). Seed will then germinate in the second spring. Such seeds have been categorised as "Two-year seeds" (Crocker, 1948), requiring a warm period in the soil to disintegrate the coat, followed by a cold period to overcome physiological dormancy.

This dormancy is a natural mechanism which tree species have adapted to allow germination over a considerable time period (Harper, 1977). This process allows for a more

guaranteed survival of the species than would 100% germination over a short period of time - i.e. a natural, or unnatural, disaster will not wipe out all individuals if only a few have germinated. If, however, a few seeds germinate occassionally, there is a much higher chance that a few individuals will survive to maturity. However, from a production point of view this is unacceptable; a high yielding, uniform crop, is desired, perhaps more on a parallel with a modern day arable crop where germination is synchronous and quick (Bewley & Black, 1994).

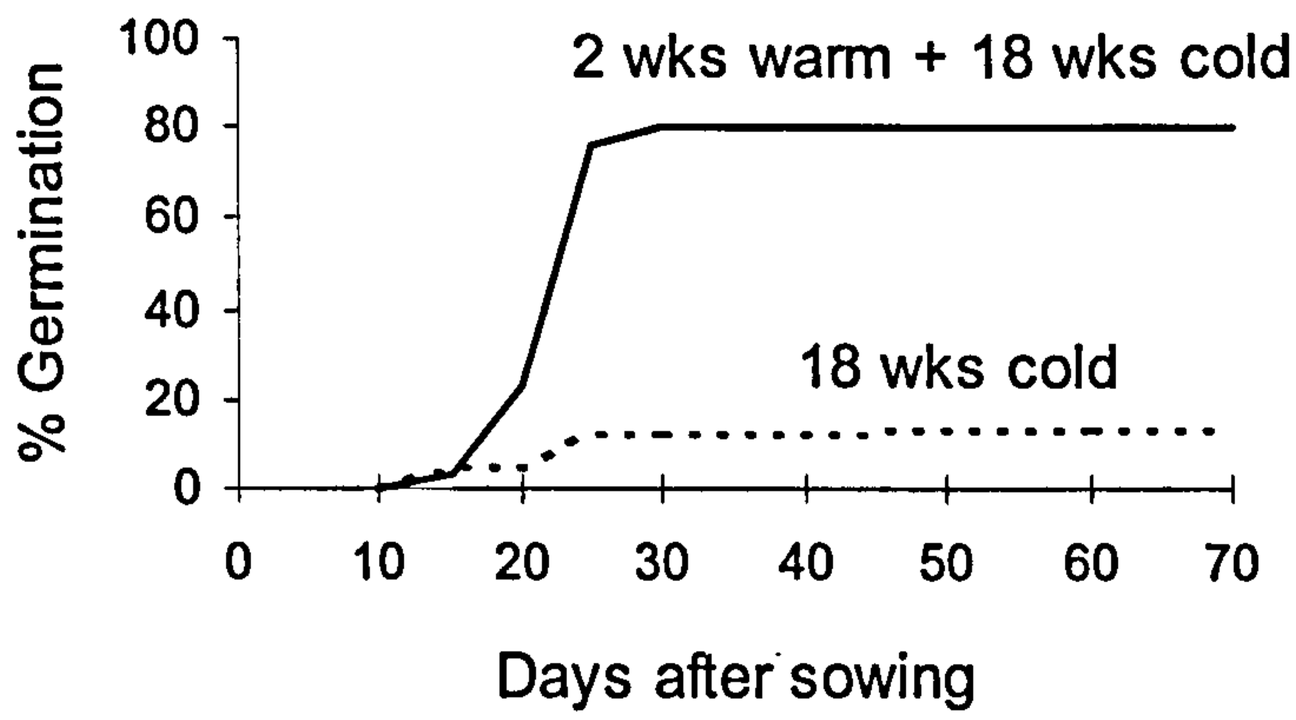
1.4.2 Traditional and novel methods for overcoming germination problems

As already mentioned the main principle in enhancing germination is to pretreat the seed in some way so as to overcome the physiological and/or physical dormancy present within any given batch. Some species also require a period of time to allow embryo maturation. *Ginkgo biloba,* for example, sheds seed with immature embryos, but these will mature during dry storage (Hatano & Kano, 1952). However most seeds with immature embryos benefit from moist storage at warm temperatures to facilitate enlargement and differentiation of the embryos (Wilbeck, 1920; Stewart & Semeniuk, 1965; Svejda & Poapst, 1972). This will result in an increased percentage germination when compared to an untreated batch and potentially more synchronous emergence allowing for a more uniform stand of trees with little or no waste during grading. However, with few exceptions commercial tree seed pretreatment relies on temperature control in moist conditions to realise the maximum percentage germination. Where species differ in these regimes is in duration of pretreatment and precise temperatures. Whereas traditional methods used ambient temperatures, modern procedures may employ controlled temperature cabinets or rooms. Whilst the concept of total temperature control may be attractive from a scientific and reproducible point of view, there is evidence, particularly in weed species, that fluctuating temperatures (as would be experienced in the natural environment) may be of considerable benefit in overcoming dormancy (Morinaga, 1926; Warington, 1936; Thompson, 1969 and 1974; Thompson & Grime, 1983). A constant 25°C, 24 hours a day (for example), is not a natural phenomenon.

Pretreatment may be as simple as storing dry seed at a suitable temperature until sowing to prevent germination in cases where dormancy does not exist. Such species include some *Acer*, *Aesculus*, *Quercus* and *Ulex*. More usual are the species which are dormant and require a warm and cold pretreatment of approximately 24 weeks, enough time from

autumn harvest to spring sowing (i.e. to coincide with the cycling of seasons), although time spans vary considerably. The ratio of recommended warm to cold varies, with extremes being 2 weeks warm followed by 18 weeks cold (e.g. *Prunus* spp.) to a recommended 40 weeks warm and 24 weeks cold for *Ilex* (Gordon & Rowe, 1982).

Warm refers to a temperature range of 20-30°C and cold to 1-5°C (Gordon & Rowe, 1982). An example of the benefit of warm and cold treatment is shown below (Figure 1.2). Only 14% germination was achieved with *Prunus spinosa* after 18 weeks cold, however when this was preceded by only 2 weeks warm, 80% germinated. The contrast in time to reach maximum germination is also obvious; 50 days for the cold only treatment and 30 days for the warm and cold combination treatment.



(taken from Gordon and Rowe, 1982)

Figure 1.2 The advantage of warm and cold pretreatment on *Prunus spinosa*, a deeply dormant, hard coated member of the *Rosaceous* family.

Substrates in which the seed is pretreated vary. This often depends upon the preference of the individual grower, although guidelines exist based upon work carried out at the Forestry research station at Alice Holt Lodge (Gordon & Rowe, 1982). Substrates commonly used are sand, peat, compost and seed in the 'naked' state (Gordon & Rowe, 1982).

The desire to increase percentage germination or to even get some difficult species to germinate at all has long been the goal of many workers. When success has been achieved it may remain confidential either by accident (passed down through the generations) or on purpose to achieve greater germination than rival nurseries or seed suppliers. This has led

to many different methods being tried to varying degrees of success; some more bizarre than practicable.

These treatments vary from physical attack on the seed coat to more subtle approaches such as the use of hormones. The following have been reported in the literature;

- Mechanical scarification involves subjecting seed to harsh abrasion with a substrate such as sand or carborundum, which, depending upon the nature of the seed batch (i.e. not uniform in individual seed size and coat thickness) can result in only a fraction of the seed having the correct treatment. The rest would either suffer physical damage to the embryo or not have enough of the coat removed (Tran & Cavanagh, 1984). Mechanical scarification is used for legume species (Todd-Bockarie *et al*, 1993) and some other tree species seed (U.S. Department of Agriculture, 1974).

- Chemical scarification is usually acid scarification, where concentrated sulphuric acid is used to burn off the hard seed coat (Blundell & Jackson, 1971 and Todd-Bockarie *et al*, 1993). This process requires great care not only because the acid is potentially very dangerous, but also because constant supervision and testing of the seed is needed throughout scarification. Care is also required to prevent the exothermic reaction from damaging the embryo. Monitoring of seed coat thickness is also required to avoid acid reaching the embryo. These factors has prevented acid scarification from becoming a widespread practice.

- Direct application of hormones (such as gibberellins and abscisic acid) proved difficult due to a lack of knowledge of effective concentrations to be applied. Breakdown, uptake and no real understanding of the complex interactions between different families of hormones and analogues within families, combined with high cost, has meant this form of enhancement of germination has never found commercial favour (Pitel & Wang, 1988; Persson, 1993).

- Alcohols have been shown to aid germination, possibly by enhancing water uptake. Verschaffelt (1912) enhanced germination in *Mimosoid* seed using alcohol, whilst Cavazza (1951) had similar success with *Gleditsia*. Acetone and organic solvents have had limited effect, being dismissed as relatively ineffective (Barton, 1947; Brown &

Booyse, 1969) except in isolated cases such as *Acacia* and *Cercis* (Barton, 1947). McKeever (1937) reported success with xylene, ether, acetone and chloroform.

- Freezing seed artificially by means of liquified gases such as nitrogen have aided germination, usually by fracturing the seed coat (Brant *et al*, 1971 and Barton, 1947).

- Hot or boiling water application to seed (especially waxy coated seeds such as those of *Acacia* species) by stripping the external barrier can result in enhanced germination (Hanna, 1984).

- Other thermal effects such as dry heat (radiation and electromagnetic waves) have been used to limited effect (Tran & Cavanagh, 1984). Microwave energy has been associated with enhanced germination (Tran, 1979).

- Fire, whether directly or indirectly by providing a heat source stimulates germination in many species, notably hard coated legumes in areas such as Australia (Beadle, 1940). The fire itself can influence germination by releasing the seed from woody inflorescences of *Banksia ornata* - a heath bush (Gill, 1975). Without the fire the inflorescences would not open (Bradstock & Myerscough, 1981).

- Smoke can contain natural 'cues' (Brown & Botha, 1993) shown to release dormancy in some species and cause germination in celery and over 65 species in South Africa - many of which have major horticultural use (Thomas & Van Staden, 1995; Brown & Botha, 1993). Van Staden *et al* (1995) and Drewes *et al* (1995) concluded that the smoke cue substitutes for red light in the germination of lettuce seeds. This mechanism is especially important in regeneration of vegetation following fire.

- Chemical effects have been reported for seed germination enhancement. Nitrate in particular has commonly been found to influence dormancy and subsequent germination. Hilton (1984), Goudey *et al* (1986) and Sexsmith & Pittman (1963) all reported stimulatory effects in germination when treated with an ammonium source, whilst Fawcett & Sliffe (1978) contradicts this. This work all focused on oat or weed species, and was partly to link fertiliser use and seed germination. No evidence has been found in the literature for a positive or negative effect for any tree species.

- Light is recommended for germination tests and is a requirement for many species such as lettuce (Borthwick *et al*, 1954) and celery (Thomas, 1975). However, whilst recommended for tree species (ISTA, 1993), few species have been shown to have an absolute requirement for light except in the specific case of ash (Bewley & Black, 1994).

- Gaseous enhancement has also been found, where ethylene was shown to stimulate witchweed germination (Eplee, 1975). Oxygen, if limited, can retard germination (Porter & Wareing, 1974), as can elevated CO_2 (Harper, 1977).

- Enzyme etching of seed coats to overcome physical dormancy has been shown in *Solanum* species (Lester & Durrands, 1984 and Lester, 1985) and *Irpex* (Noguchi *et al*, 1978). Yambe *et al* (1992b) improved germination of Rose achenes using a macerating enzyme preparation. Interestingly, only one hybrid rose was used (reported) and no subsequent work has been published.

- Microbial protectants may also be a treatment to enhance germination by preventing loss from decay during storage and initial stages of growth. Tree seed germinated in sphagnum moss showed reduced levels of mould fungi, ensuring good germination (Lin, 1995). Hartz and Caprile (1995) used a microbial coating on maize to the same effect whilst Howell (1991 and *et al* 1993) prevented damping off fungi on cotton.

- Other potential agents proposed by the author but not found in the literature include milder acid and alkali washes to remove waxes, oxidising agents, detergents and tannins.

1.5 *Rosa corymbifera* 'Laxa'

1.5.1 The history of *Rosa corymbifera* 'Laxa'

Rosa corymbifera 'Laxa', formerly *Rosa dumetorum* 'Laxa' is the main rootstock used in British rose production. Whilst demand for this species has dropped due to an overall fall in rose production across the country (Wheatcroft Nurseries, 1994), a closely related species, (*Rosa canina* 'Inermis'), is used extensively in Dutch rootstock production (Besten (1995), personal communication).

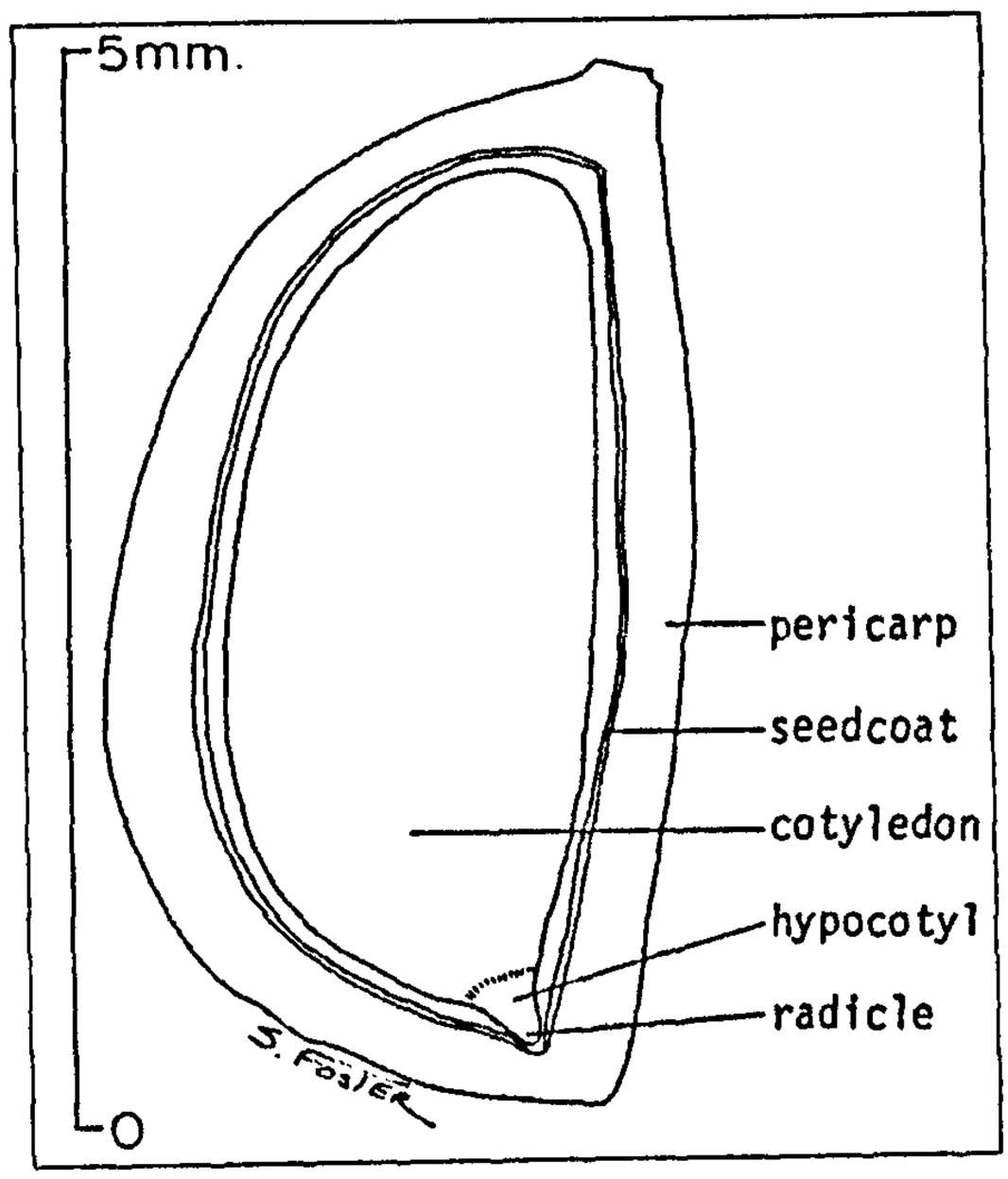
During the nineteen seventies and eighties, *Rosa corymbifera* 'Laxa' production was extensive and could produce a high yield and profit (Roberts, 1979). One independent rose producer, Wheatcroft Nurseries, alone cultivated a 9 acre plot of stock bushes. This equated to a yield of approximately 3600kg of seed per annum (approx. 2.55×10^8 individual seeds). However the dramatic decline resulted in these being grubbed out in 1995.

In 1982 MAFF produced a leaflet detailing cultivation of *Rosa corymbifera* 'Laxa' from seed with a view to producing a seed crop (which superceded Roberts, 1979). It described strict practices for sourcing seed material, extraction of seed, seed treatment and storage. Seedbed preparation, disease control and seedling harvesting and grading were also detailed. Three treatments were suggested to overcome dormancy - natural stratification, temperature controlled storage (warm/cold) and acid scarification. Maximum germination percentages for these three treatments were given as 7-15, 25-50 and 65-75 respectively. Each method has drawbacks, either due to low percentage germination or problems in the technique itself. The highest germination was achieved with acid scarification, however this involves handling many litres of concentrated sulphuric acid - which is very corrosive and should be avoided if at all possible due to the high risk to the handler of the acid during use and the environmental consequences of disposal (difficult to justify under COSHH regulations).

It would therefore be of great advantage and economical benefit if a new or alternative pretreatment for *Rosa corymbifera* 'Laxa' could be implemented which combined the high percentage germination of acid scarification with the simplicity of natural stratification. Such a pretreatment would have universal appeal from the commercial seed houses which

treat tons of seed to the individual nurseries treating a only a few grams. A pretreatment which works successfully for *Rosa corymbifera* 'Laxa' might act equally well with similar seeds, especially within the *Rosaceous* family.

Research into this species found that little work had been done to establish the anatomy of the seed. Only two references were found, a poor scanning electron micrograph of *Rosa hybrida* L. (Gudin *et al*, 1991) and a line drawing of *Rosa setigera* (Gill & Pogge, 1974), shown below, which provides only the general structure.The 'seed' of *Rosa* spp. is actually an achene borne within a fleshy, berry like hip (Gill & Pogge, 1974). However, for the purposes of this thesis the achene will be referred to as a seed.



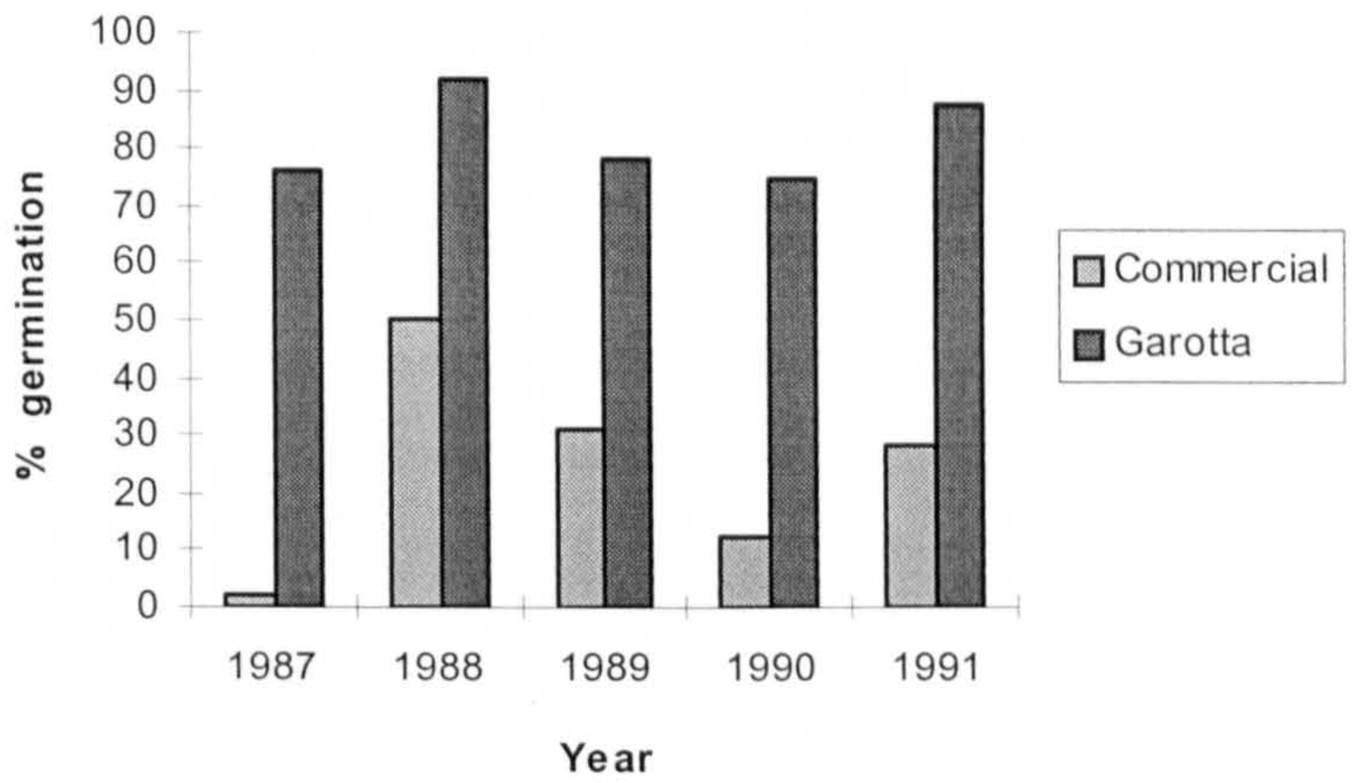
taken from Gill and Pogge (1974)

Figure 1.3 Line drawing of a cross section of a *Rosa setigera* seed (one of only two Rose species found illustrated during searches of the literature). It lacks detail and the labelling is ambigious.

One of the first aims of this research was therefore to produce a detailed record of the anatomy of *Rosa corymbifera* 'Laxa' to give insight into how mechanisms may be used to overcome the germination problems (chapter 3).

1.5.2 A new pretreatment

It was the lack of evidence on the natural mechanisms of alleviating hard coated seed dormancy (see section 1.2) which initiated work at Writtle College in the late eighties. Various items and compounds were initially introduced to the pretreatment mix of moist vermiculite and seed. Amongst these items were rotten wood, fertiliser and a compost activator sold commercially as Garotta. A variety of tree species were involved, but it was found of all the combinations of species and additions, that the compost activator and *Rosa corymbifera* 'Laxa' showed a marked enhancement on seed germination. This was subsequently repeated and the same result obtained (figure 1.4) (Cullum *et al*, 1990). It was therefore decided to use *Rosa corymbifera* 'Laxa' as the 'model' to be studied throughout this thesis.



from Cullum *et al* (1990) and Cullum (personal communication).

Figure 1.4 The percentage germination of *Rosa corymbifera* 'Laxa' following the commercial and Garotta pretreatment.

Detailed research was therefore required to study this phenomenon with *Rosa corymbifera* 'Laxa' and apply the treatment to other hard coated species. The technique solves the two requirements of softening the seed coat and still allowing adequate time for a cold treatment prior to the first spring sowing. Traditional warm/cold treatments commence in the first spring following harvest and therefore continue over to the second spring, up to 18 months after harvest. This can pose problems of long term storage, which in some species

could induce secondary dormancy, especially in dark conditions (Bewley & Black, 1994) and increase the proportion of non viable seed.

Garotta is sold at most retail outlets concerned with the horticultural business, including garden centres and DIY shops (personal observation). It has a dry, grainy appearance resembling a grey sand. It is promoted to apply to compost heaps to "....make rich organic compost in weeks" and has the largest market share of all the activators. The exact composition is not know as it is a trade secret, however there can be no magic ingredient associated with it as the cost is only £3 per Kg.

It would therefore be an ideal addition to pretreatment mixes due to its low cost and relatively small amounts used. Garotta also has the benefit of being a readily available product with no associated risks or hazards. It was estimated that 1 Kg would treat the whole rose rootstock seed in the UK per year!

1.6 Overall aims of the thesis

- To develop a simple and reproducible commercial pretreatment for *Rosa corymbifera* 'Laxa' in the laboratory.

- To describe the detailed anatomy of *Rosa corymbifera* 'Laxa' seed.

- To investigate the effect of the addition of Garotta on the germination of *Rosa corymbifera* 'Laxa' following pretreatment.

- To identify the origin of microorganisms entering the pretreatment and to study their role, if any, in the germination of *Rosa corymbifera* 'Laxa'.

- To develop a commercially acceptable pretreatment to overcome dormancy and enhance germination consistently for hard coated native tree species.

- To ascertain that laboratory germination rates are comparable with those obtained in the field.

2. ENHANCEMENT OF NATURAL GERMINATION

Regeneration of native trees has continued naturally for thousands of years without the intervention of man. Germination rates may be low and emergence sporadic, but tree seed can germinate quite adequately in the wild. It must therefore follow that in the natural environment seeds can lie dormant whilst natural processes occur which result in germination. Perhaps the most important external factors are the prevailing temperatures and presence of microbes. Whilst some authors cite natural breakdown of seed coats in their texts (outlined in section 1.2), no experimental work is presented or referenced to back up these logical assumptions.

The purpose of this chapter is to show the effectiveness of running a 'natural' pretreatment on *Rosa corymbifera* 'Laxa' with regards to germination and to ascertain whether microorganisms are present. If microorganims are present then it is important to know where they come from, and equally, what effect on germination their enhancement or elimination may have.

Aims - To develop a simple and reproducible commercial pretreatment *for Rosa corymbifera* 'Laxa'. To identify the source, if any, of microorganisms entering the pretreatment.

Objectives - to develop a simple laboratory based commercial pretreatment based upon current commercial practice in the industry.
To ascertain whether the *Rosa corymbifera* 'Laxa' seed used in the pretreatment has a microbial loading.
To determine whether any of the constituents of the pretreatment are microbially loaded.
To obtain an understanding of the constituents of Garotta.
To enhance the natural germination described in section 2.1 by including Garotta in the pretreatment of *Rosa corymbifera* 'Laxa'.
To monitor temperature and pH of the commercial and Garotta pretreatments during the warm period.

2.1 Mimicking natural germination

To investigate the natural association of microbes with seed germination a standard 'commercial' pretreatment was designed upon which to base comparisons with other treatments. Natural germination conditions and standard nursery practice were taken into consideration when choosing the media and the incubation criteria for the 'commercial' pretreatment.

Objective - to develop a simple laboratory based commercial pretreatment based upon current commercial practice in the industry.

2.1.1 Materials and Methods

Rosa corymbifera 'Laxa' hips were harvested from the stock bushes at Writtle College, Chelmsford, Essex and from stock plants at Wheatcroft Nurseries, Nottingham. Hips were picked when red and firm and manually crushed prior to soaking in sterile tap water. Any damaged or diseased hips were discarded. After three days the achenes were recovered from the softened fruit through a series of sieves. Achenes were washed in running tap water before being allowed to air dry on the bench. If the seed were to be stored it was placed in 'breathable' bags and kept at room temperature. All other tree seed was purchased already extracted and cleaned from Forestart, Hadnall, Shropshire.

Tree seed is pretreated in many different combinations of substrate depending upon grower preference and species. However, peat, sand and vermiculite are the most commonly used (Gosling & Aldhous, 1994; Gordon & Rowe, 1982; D. Fordham, personal communication). The decision was made to use vermiculite. This is an inorganic medium with good water retention (essential to keep the seed moist during pretreatment (Gosling & Aldhous, 1994; Gordon & Rowe, 1982)).

A pretreatment mixture was made up from several components depending on treatment. Vermiculite (horticultural grade, kindly donated by Sinclair Horticulture) was taken where possible from a new, sealed bag for each experiment. Tap water was added to the vermiculite to give a final water holding capacity of 70%. This was previously calculated by soaking samples of vermiculite to 100% water holding capacity, draining on a sand

suction table, and drying back to 0%. The difference in mass represented the amount of water required to give 100% water holding capacity. This equated to 915g of water to 600g vermiculite. To eliminate any microbes the moist vermiculte was then autoclaved and allowed to cool before use.

Standard proportions of seed and vermiculite were used as in the protocol of Cullum *et al*, (1990). 10g moist seed (soaked for 24 hours in sterile tap water) was mixed with 25g moist vermiculite. This represented the control or commercial treatment, designed to mimick the natural conditions.

Material used for laboratory experiments was set up in rigid, 2*l* containers, whilst that destined for field trials was treated in plastic bags (similar to the commercial grower). At no point during the pretreatment were boxes or bags sealed. There was always some opening to facilitate air movement, and therefore the potential for air microflora to enter the system. All treatments underwent a standard pretreatment of 12 consecutive weeks at 25°C followed by a further 12 weeks at 4°C unless otherwise stated. Twice a week during the warm period the contents of the bags/containers were made up to the original set up weight with sterile tap water and shaken (or stirred in the case of the boxes) to allow aeration. This was reduced to once a week during the cold period.

After the specified pretreatment period, four lots of 100 seeds were taken for germination tests from each replicate. This number conforms to the specification recommened by the International Seed Testing Association (ISTA) guidelines (1993). Laboratory testing was carried out in petri dishes whilst field trials were conducted in pre-prepared seed beds at Oakover Nurseries, Kent.

Some historical data exist for *Rosa corymbifera* 'Laxa' germination based on previous laboratory work. This does not constitute such rigorous sampling (i.e. before ISTA guidelines were followed) but will be shown to illustrate variation in control germination, and in section 2.3, enhanced germination.

2.1.2 Results

The graph below (figure 2.1) shows the percentage germination of *Rosa corymbifera* 'Laxa' following the 'commercial' pretreatment for the years 1992 - 1996. The tests carried out since 1992 followed the ISTA guidelines whereby at least four lots of 100 seeds were tested from each replicate of each pretreatment. Prior to this smaller numbers were used (Cullum *et al*, 1990). The 'natural pretreatment' is identical to commercial pretreatment, although different nurseries use different media. The term commercial pretreatment will therefore be used for the rest of the thesis to identify this pretreatment.

Whilst germination is variable each year, the overall problem is that the number is low. In bad years the 'natural' pretreatment results in only a few percent germinating, which is unacceptable to the grower.

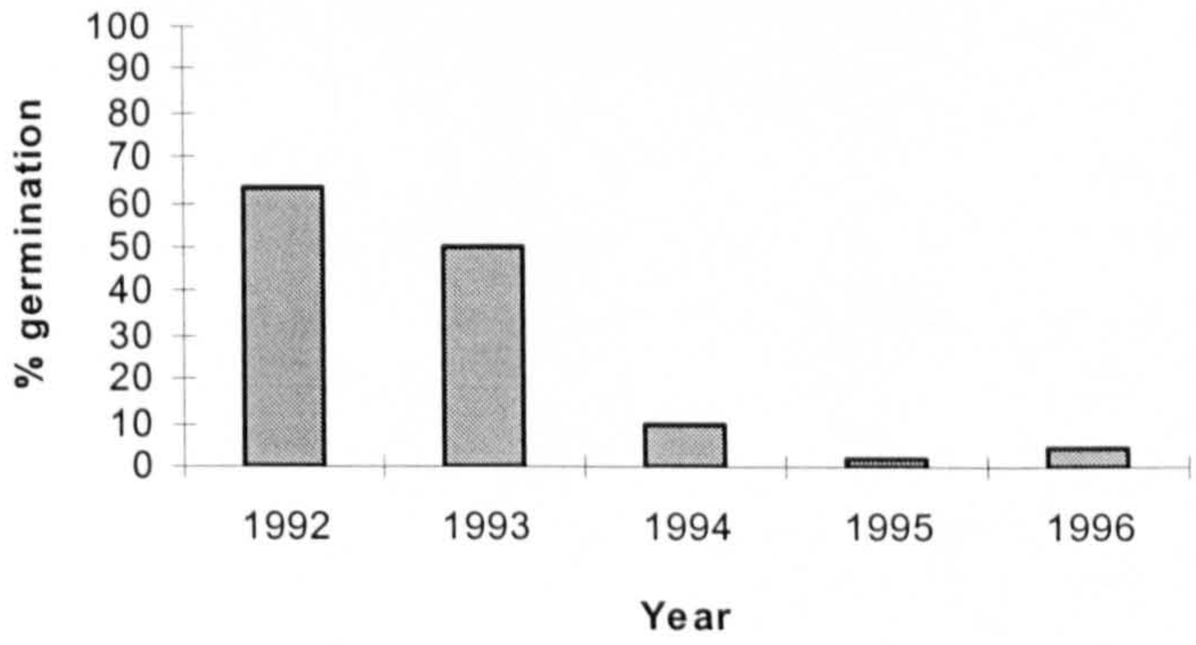


Figure 2.1 The percentage germination of *Rosa corymbifera* 'Laxa' following the commercial pretreatment from 1992 to 1996 when ISTA guidelines also apply.

2.1.3 Discussion

Germination is very variable from year to year and unacceptably low to a grower in the majority of the 5 year period. 1995 proved to be the worst year to date, with only 2% of the seed germinating. This contrasts with a relatively good 63% in 1992. However the trend is of low percentage germination. In the natural environment this would be expected as the seed would germinate over a long period of time.

Taking into consideration the work of Cullum *et al* (1990), the same trend of low germination in the commercial pretreatment is also true (see figure 1.4). Despite the figures of Cullum and co-workers being on small numbers of *Rosa corymbifera* 'Laxa' seed, the same result is observed. A grower would not find such numbers acceptable and would strive to increase germination percentage.

2.2 Microbial loading and seed

Objectives - to ascertain whether the *Rosa corymbifera* 'Laxa' seed used in the pretreatment has a microbial loading; to determine whether any of the constituents of the pretreatment are microbially loaded.

2.2.1 Materials and Methods

1g samples of seed were taken from various stages along the protocol of extracting seed from freshly picked hips. These were;

- Intact hips (into 9mls sterile tap water from the bush).
- seed aseptically excised from the hip.
- seed sampled every 24 hours during the extraction procedure.
- seed following extraction, washing and drying.

The stages in harvesting and extraction are summarised below (figure 2.2).

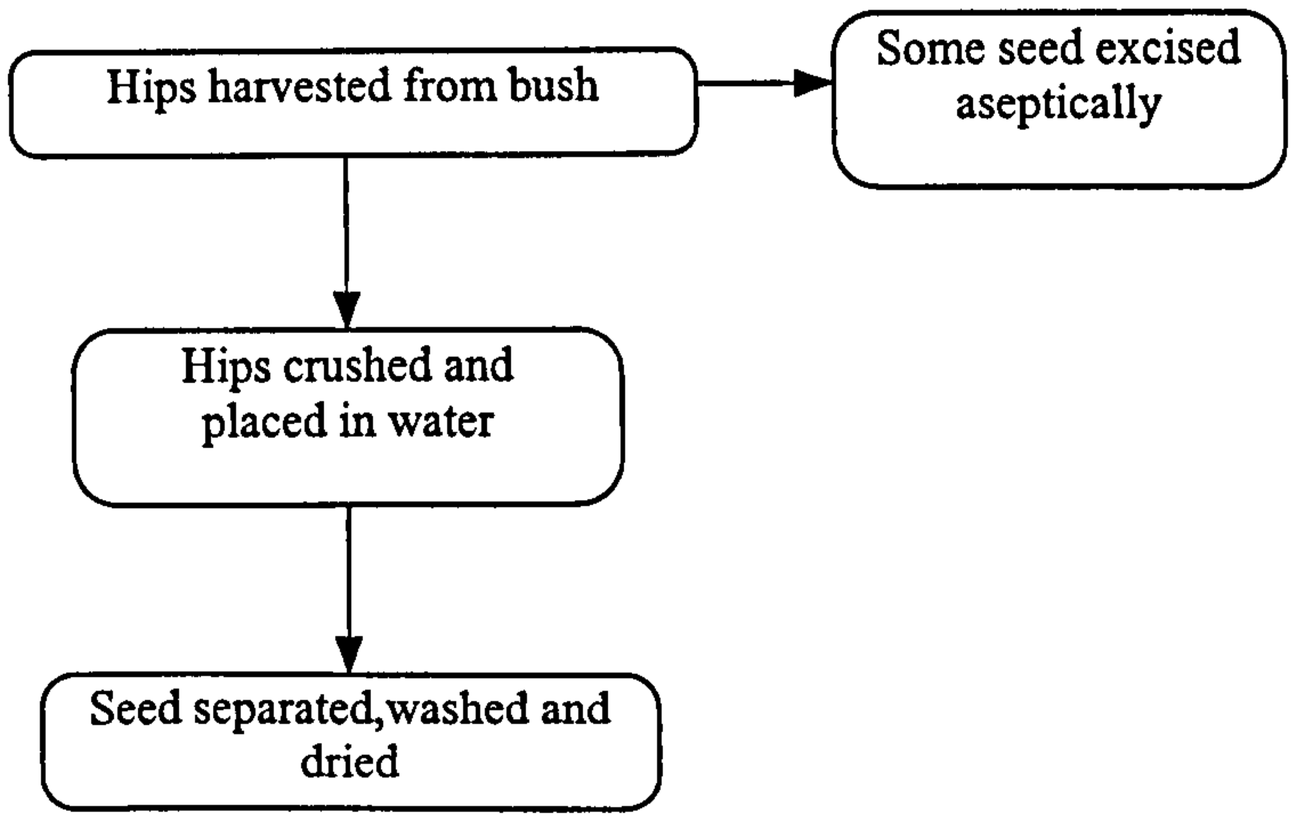


Figure 2.2 Flow diagram showing the steps from harvesting *Rosa corymbifera* 'Laxa' hips to the final dried seed product. Samples were taken at every step to check for microbes.

The 1g sub-samples (or whole hips) were placed into 9mls sterile tap water, shaken for 5 minutes and spread plates made from 100µl of the resulting wash. Pre-prepared agar plates of nutrient agar (NA) for bacteria and potato dextrose agar (PDA) for fungi were used.

Sub-samples of 10 seeds were aseptically placed on pre-prepared agar plates, again NA and PDA. All plates were incubated for 48 hours at 25°C and colonies counted.

The vermiculite, sterile tap water and Garotta* were also sampled and spread plates made of the washes. 1g of each was shaken in 9mls sterile tap water and 100µl plated out. (*Garotta is used in later experiments, but was introduced into section 1.5.2.)

2.2.2 Results

The results of the microbial loading experiments are shown in figures 2.3 and 2.4. Counts are expressed as colony forming units per gram sample. It can be clearly seen in figure 2.3 that whilst no fungi or bacteria were found in the vermiculite, water or Garotta, a microbial loading was found on the seed being used in the experiments.

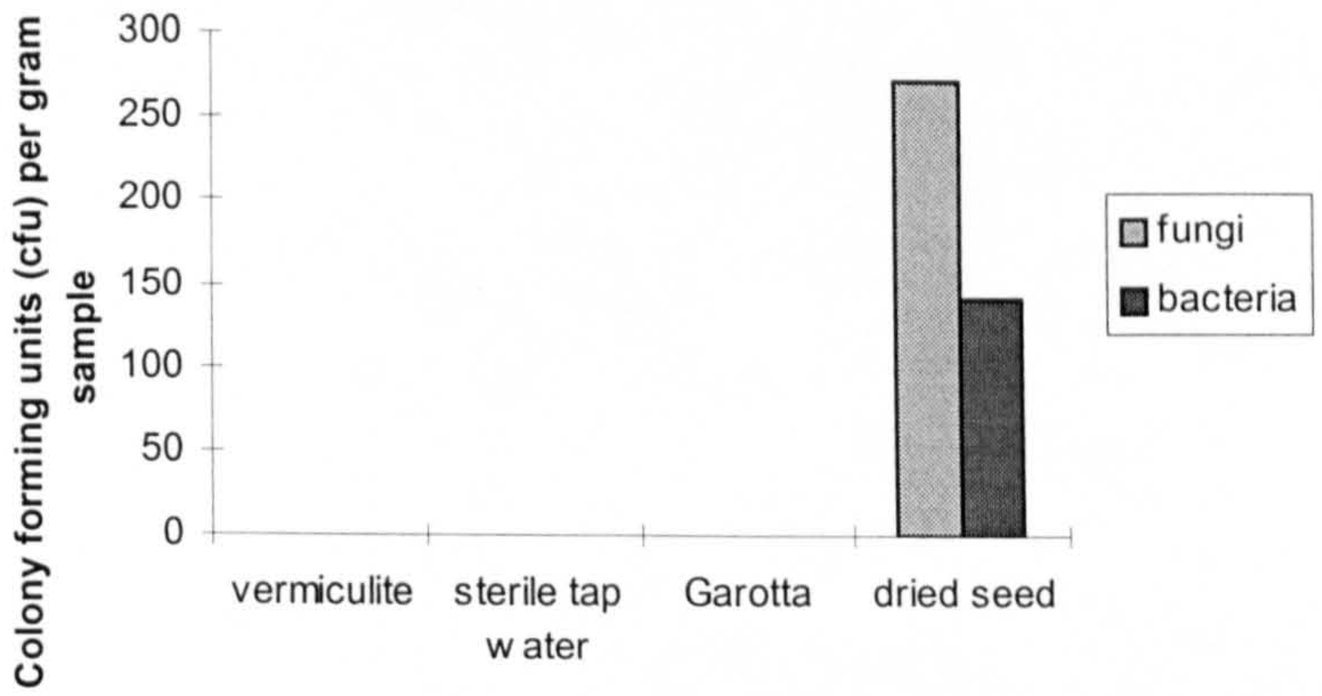


Figure 2.3 Microbial loading of the constituents of the pretreatment mix.

Figure 2.4 (overleaf) represents the microbial counts (note the scale is a $\log_{10}$ scale) made from sampling *Rosa corymbifera* 'Laxa' seed at different times during the extraction procedure. Seed excised from the intact hips was sterile although washings taken from the outer surface of the hips were found to contain fungi and bacteria. Following manual crushing and addition of water, the seed was then found to have a microbial loading similar to that of the intact hips.

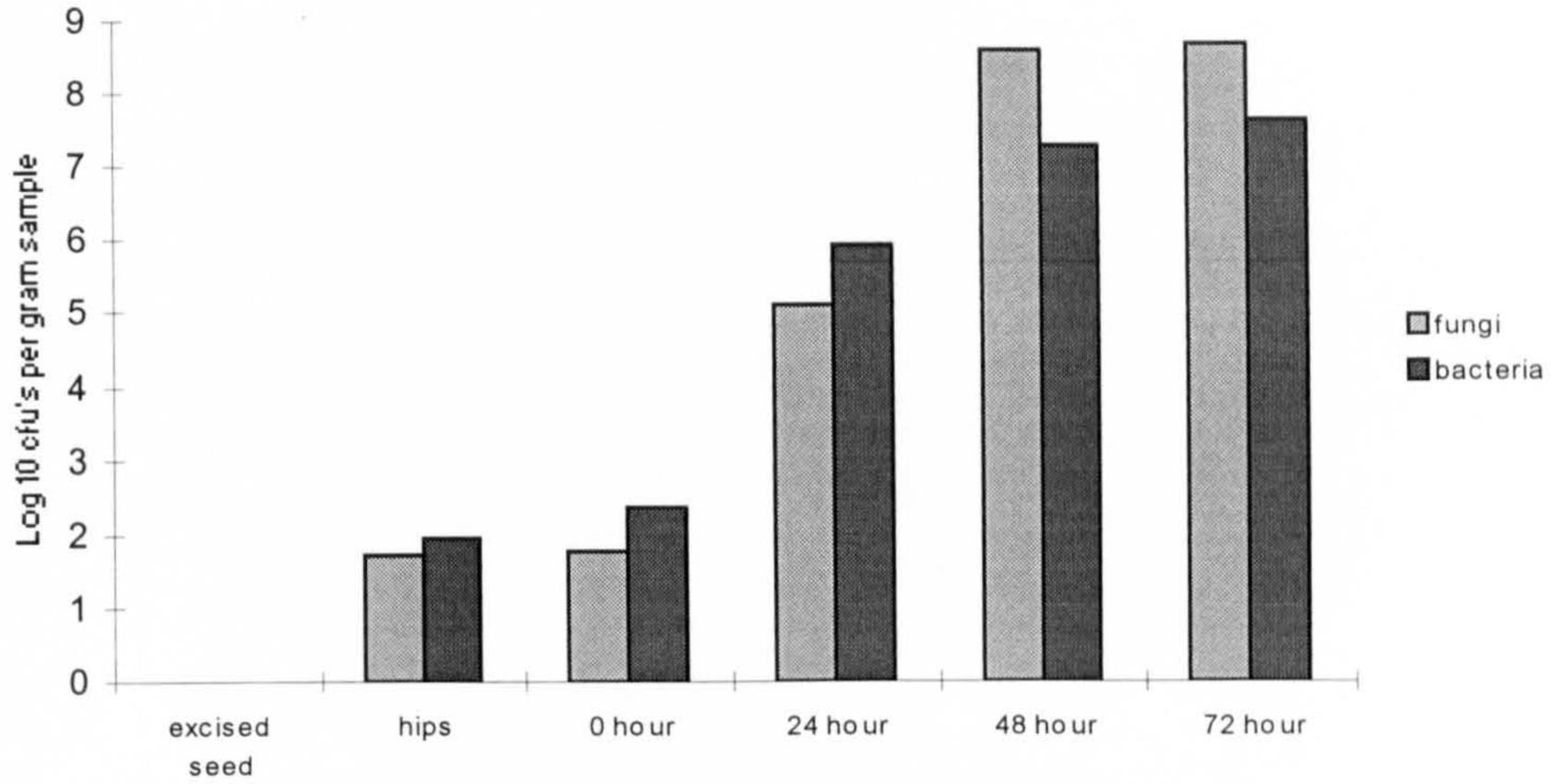


Figure 2.4 Microbial loading on the seed during the different stages of extraction from the hips.

After 24 hours softening in water, the numbers of microbes increased dramatically compared to the 0 hour washing of seed. This microbial loading then increased at 48 and 72 hours. At the 72 hour time point the seed was separated from the hip material and washed several times prior to drying. The microbial loading following washing and drying (excised seed) was greatly reduced compared to that found on the seed during extraction (see figure 2.4, above).

2.2.3 Discussion

The results clearly show that a natural microbial loading enters the pretreatment mix, and that it comes from the seed. The other constituents are sterile. The selection of vermiculite was partly to ensure a sterile substrate could be used to eliminate this component of the pretreatment as a potential variable in the experiment. It is inert and remains stable during autoclaving.

It can also be seen from the results that the *Rosa corymbifera* 'Laxa' seed is sterile within the freshly picked hips, and that the outer surface of those hips carries some microbes. These microbes then multiply during the softening of the hips, in a similar fashion to fermentation. This 'transfer' of microbes onto the seed would be most effective once the hips were physically crushed for extraction and put into the water. It is at this point that the seed becomes 'microbially loaded'.

The numbers of microbes found on the dry seed after washing (following separation from the hip material) were low compared to those during extraction in the wet state. This can be accounted for by the vigorous washings in cold water the seed goes through whilst being recovered from the hip material, and the subsequent desiccation on the bench. This drying of seed under ambient conditions is how commercial seed houses treat tree seed.

2.3 Enhancing natural germination

Garotta is a proprietary compost activator which is used to enhance composting by encouraging microbial growth (Sinclair Horticulture - product description). It could therefore be added to the pretreatment mix described in section 2.1.1 to increase microbial growth during the warm incubation. Any effect on germination would be evident when compared to the commercial pretreatment. Garotta was used by Cullum *et al* (1990) to enhance germination in *Rosa corymbifera* 'Laxa' , although the mechanism for its action was not researched.

Objectives - to obtain an understanding of the constituents of Garotta. To enhance the natural germination described in section 2.1 by including Garotta in the pretreatment of *Rosa corymbifera* 'Laxa'. To monitor temperature and pH of the commercial and Garotta pretreatments during the warm period.

2.3.1 Materials and Methods

The manufacturers of Garotta, Sinclair Horticulture, were contacted and asked for information regarding the constituents of Garotta, and analysis was carried out in the laboratory using the methods described by MAFF (MAFF, 1986). Courtaulds Chemicals also provided some data.

Garotta was added to the pretreatment mix described in section 2.1.1 at a rate of 1g Garotta to 10g moist seed. The commercial pretreatment was as described in section 2.1.1. Temperature was monitored in three commercial and three Garotta pretreatments. Ambient temperature within the incubator was also monitored. The pH of the pretreatment mixes was measured at setup and then after 2, 4, 6, 9, 15, 22 and 28 days, and weekly until the cold period of incubation. Both the the commercial and Garotta pretreatments were assessed, as well as the equivalent pretreatments, without the addition of the seed.

2.3.2 Results

The response from the manufacturers of Garotta to its formulation was limited. However, they did volunteer that sulphate of ammonia (ammonium sulphate), lignite and limestone were the major components of Garotta (Turner, 1993, (personal communication)). The separate 'approximate' analysis carried out by Courtaulds Chemicals produced a list (by weight) consisting of sand (38%), limestone (37%), water soluble ammonium salts (22%) and carbonaceous lumps (3% by ashing) (Courtaulds Chemicals, personal communication).

Analysis as determined in the laboratory is tabulated below (table 2.1). The water soluble ammonium salts consisted of ammonium sulphate (Turner, 1993, pers. comm.) but only the nitrogen fraction was analysed due to limitations in the sulphate analysis available.

Table 2.1 The results of the analysis carried out in the laboratory using the MAFF techniques for each component listed.

Component analysed	**Result**
pH	7.4
Conductivity	134 000 µS/cm
Extractable Phosphorus	365 mg/l
Extractable Potassium	2503 mg/l
Extractable Magnesium	68 mg/l
Extractable Calcium	6060 mg/l
Nitrate (Nitrogen)	361 mg/l
Extractable Cadmium	0.32 mg/l
Extractable Copper	1.6 mg/l
Extractable Lead	2.8 mg/l
Extractable Nickel	none detected
Extractable Zinc	4.8 mg/l

The effect on germination of *Rosa corymbifera* 'Laxa' with the addition of Garotta is shown in figure 2.5. The results of the commercial pretreatment are shown as comparison from section 2.1.2.

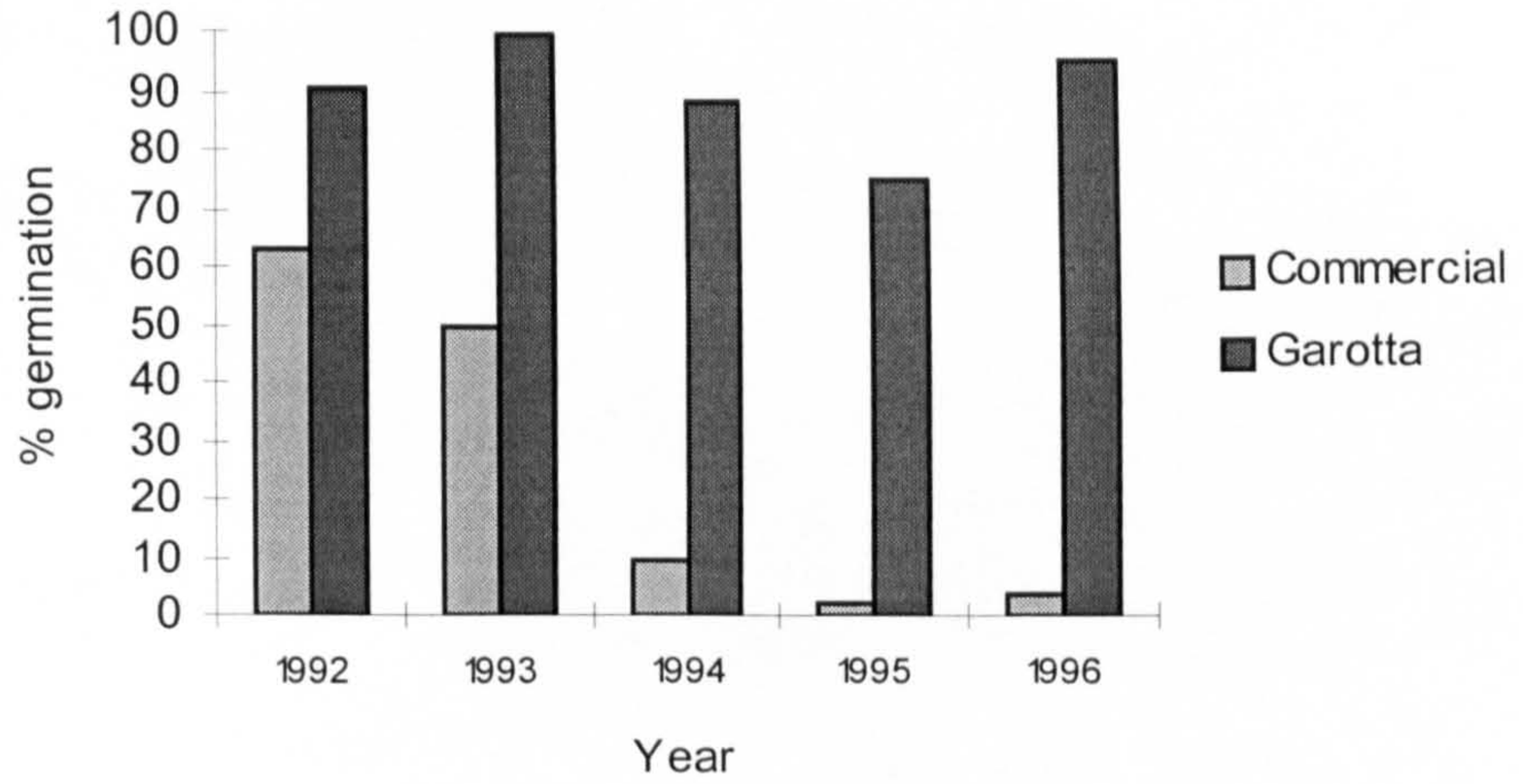


Figure 2.5 Germination following pretreatment of *Rosa corymbifera* 'Laxa' under commercial conditions and with the addition of Garotta.

The graph shows quite clearly the advantage in adding Garotta to the pretreatment mix. Germination is consistently higher every year over the commercial pretreatment. Not only is percentage germination higher, it is more consistent. The variation in germination is between 2% and 63% for the commercial pretreatment, compared with only 75 to 99% in the Garotta treatment.

The results of the temperature and pH studies can be found in appendix 2.1. There was no difference found between ambient temperature and either pretreatment (commercial or Garotta). The fluctuations in the 'ambient' temperature of the incubation were identical to those in the commercial and Garotta pretreatment. The pH was also consistent for the pretreatments. There was no difference in pH of the media for any of the pretreatments.

2.3.3 Discussion

The analysis carried out on Garotta by Courtalds Chemicals corresponds well with the information given by the manufacturers. Sulphate of ammonia corresponds to soluble ammonium salts, limestone as calcium carbonate and lignite is a 'brown coal of woody texture' which would ash as carbonaceous lumps. Sand would be present as an extra bulking agent. Neither communication indicated any microbial input to Garotta, and experimentation has also shown this to be the case (section 2.2.2). The laboratory analysis also found high levels of calcium and nitrate corresponding to the ammonium salts and calcium carbonate. Other elements found were consistent with a high soluble salt content (phosphorus and potassium) and some trace elements.

The addition of Garotta changed the germination characterisics of *Rosa corymbifera* 'Laxa' in two very important ways when compared to the commercial pretreatment. Firstly, the percentage germination increased every year over the control. Secondly the great variation seen from year to year in the commercial pretreatment was diminished with the addition of Garotta. Whereas the lowest germination was 2% in the commercial pretreatment, it was 75% in the Garotta pretreatment. The highest recorded germination in the commercial pretreatment was 63%, but 99% was achieved with Garotta. These figures also show the difference in range of germination within *Rosa corymbifera* 'Laxa'. A spread of only 24% covered 10 years germination data with a Garotta addition, whereas the commercial pretreatment showed a variation of 61%.

The monitoring of temperature and pH showed no difference between ambient and the pretreatments (for temperature) or between any of the pretreatments for pH. This work showed that any effect found during pretreatment was not due to any temperature increases (such is found in composting) or due to pH changes which could cause membrane changes in the seed.

2.4 Summary Discussion

A simple and reproducible commercial pretreatment was achieved in the laboratory, which could be directly used on a commercial scale in the industry. It is evident from the work presented in this chapter that under commercial conditions *Rosa corymbifera* 'Laxa' seed will germinate in a sporadic manner from year to year, often in very low numbers.

Microorganisms have been shown to be present in the pretreatment mix, and originate from the seed which was used in the experiments. The initial source of microbes was shown to be the environment, specifically the outer surface of the hips. These multiplied during separation of the seed from the hips, and during seed extraction 'loaded' the previously sterile seeds.

The addition of Garotta promotes total germination percentage, i.e. more seeds germinate after Garotta pretreatment than without (commercial pretreatment). Garotta also greatly decreases the variation between years of percentage germination. A much narrower band of germination percentage was found with the Garotta pretreatment than the commercial pretreatment.

Garotta would not appear to contain any 'magic' ingredient, in fact it is made up from very common and easily available raw materials. This is reflected in the price, in that if expensive additives were included, it would cost more that £3 per kilogram. It also has no detectable microbial element as found in section 2.2.2.

Temperature and pH did not alter during the warm period of pretreatment in either the commercial or Garotta pretreatments when compared to the ambient temperature. A composting effect was not found with the seeds, and the possibility of large pH changes affecting cell membrane integrity within the seed is therefore unlikely.

3. PHYSICAL CHARACTERISTICS OF SEED DURING PRETREATMENTS

When the literature was searched little material was found detailing the structure and anatomy of *Rosa corymbifera* 'Laxa' (see section 1.2). It was therefore deemed appropriate to look closely at untreated *Rosa corymbifera* 'Laxa' seed and then compare the structure with seed which has undergone the different pretreatments.

Aims - To study in detail the structure of untreated *Rosa corymbifera* 'Laxa'. To identify any differences in physical characteristics of the seed following commercial and Garotta pretreatments.

Objectives - to gain a clear understanding of the anatomy of *Rosa corymbifera* 'Laxa' and to produce a structural drawing showing the detailed structure of the seed.
To identify any physical differences between commercial and Garotta pretreated seeds of *Rosa corymbifera* 'Laxa' following the pretreatments.
To use scanning electron microscopy to ascertain the presence of microorganisms on *Rosa corymbifera* 'Laxa' seed.

3.1 Untreated *Rosa corymbifera* 'Laxa'

Objective - to gain a clear understanding of the anatomy of *Rosa corymbifera* 'Laxa' and to produce a structural drawing showing the detailed anatomy of the seed.

3.1.1 Materials and Methods

Hips were taken from the stock bushes at Writtle college. Samples were then dissected and seed extracted from the hips. These were air dried and examined using light and scanning electron microscopes.

Samples of hips and seed were examined and photographed prior to closer inspection using microscopes. A stereo light microscope was also used to examine samples of seed. Seed was stained with acidified phloroglucinol (see appendix 3.1 for method) to show areas of lignin deposition. Cellulose was also stained for using Gram's iodine and sulphuric acid (see appendix 3.1 for method). Photographs were taken on Fujichrome RD100 slide film, using a Pentax Program A camera. Seed was also viewed using a scanning electron

microscope (Cam Scan 3-30 at 15kV). Samples without any preparation were found to charge too quickly for any meaningful results to be obtained. Charging occurs on samples which have poor conductivity - the effect being a build up of electrons on the specimen resulting in a bright glowing making the sample impossible to view.
N.B. Plates 3.31, 3.32 and 3.33 were taken using a Jeol 6300 Win Sem at 20kV.

In order to overcome this charging seed was dried down under vacuum before being mounted on aluminium stubs using conductive adhesive tape. The samples were then gold splutter coated (Nanotech SEMPREP 2) in a 10^{-4} Mbar vacuum before being viewed. Samples of whole, cross and longitudinal sectioned seed were used.

3.1.2 Results

The results to this section are presented below as plates with appropriate explanations below.

Plate 3.1 Ripe *Rosa corymbifera* 'Laxa' hips on the stock bushes at Writtle College.
The 'typical' *Rosa corymbifera* 'Laxa' hip is deemed ripe when red and firm. This occurs around September, but varies depending upon the prevailing environmental conditions. It is when the hips are at this stage of development that they are picked to extract seed.

Plate 3.2 Two dissected *Rosa corymbifera* 'Laxa' hips (Mag^{n} x2).
The two halves on the left were from one hip, the two on the right from a second example. The arrangement of the seeds within the hips can be seen clearly. When dissected the seeds (achenes) are found to be tightly packed into the hip.

Plate 3.3 A single dissected *Rosa corymbifera* 'Laxa' hip (Mag^{n} x2).
The seeds have been removed from the hip to show the average number of seeds per hip. Hips were found to contain an average number of 19 seeds (achenes) (see appendix 3.2 for calculation based on 20 hips).

Plate 3.4 Air dried *Rosa corymbifera* 'Laxa' seed.

This is the commercial product i.e. the form in which *Rosa corymbifera* 'Laxa' would be purchased. *Rosa corymbifera* 'Laxa' has relatively small seeds compared to many tree species, with approximately 65-70 000 individuals per kilogram (air dried, authors' calculation). Each seed measures approximately 3-4mm in length and 2-3mm in width. Shape varies within any given batch. Plate 3.4 shows the relative size of *Rosa corymbifera* 'Laxa' seed.

Plate 3.5 Magnified *Rosa corymbifera* 'Laxa' seed (air dried).

This plate shows the variety in shape found amongst a sample of seed. Two distinct shapes can be seen, labelled A and B.

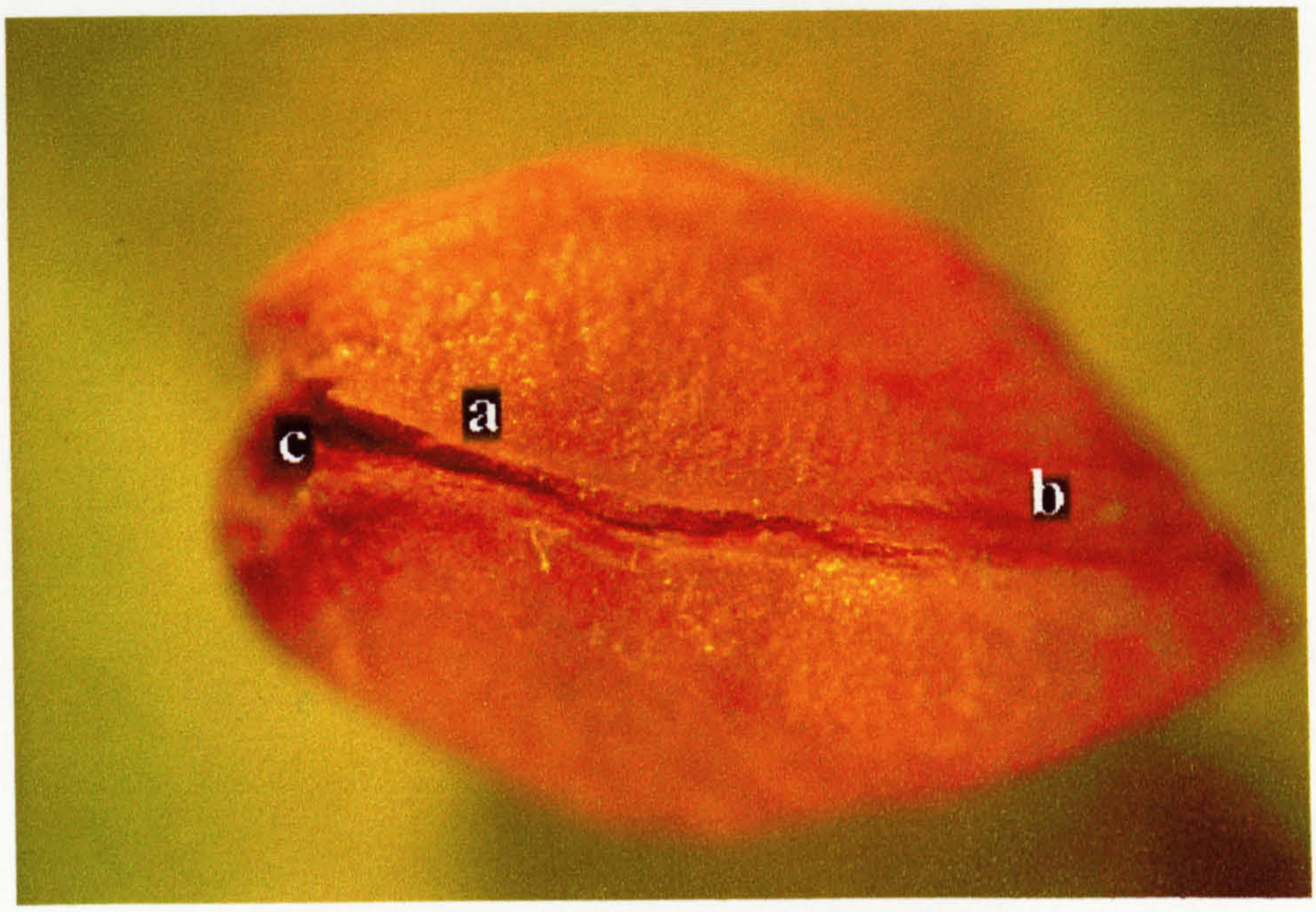


Plate 3.6 A single untreated *Rosa corymbifera* 'Laxa' seed (Mag^{n} x24).
The dark line running from the left to the right (a to b) is the suture (where the seed splits open prior to germination). The outer papery surface has pulled away along the line of the suture, hence it appears split when it is not. The rough end (c) is the hilum which is the remaining scar from when the funiculus (stalk of the ovule) abscised from the ovule. This is also a weak point and would be the first area susceptible to water or microbial attack. The extreme left and right of the seed is out of focus due to the limited depth of field possible for this plate.

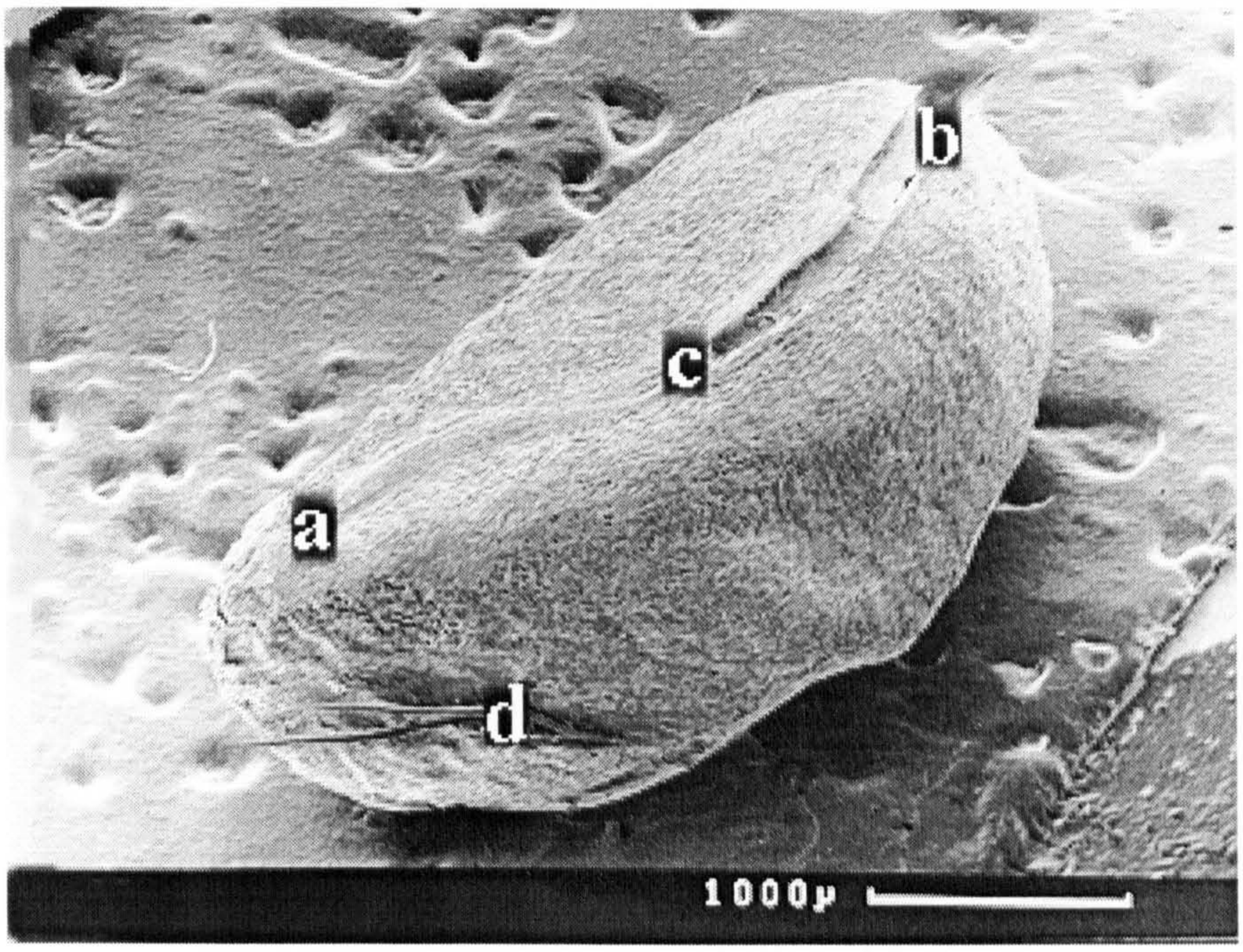


Plate 3.7 An SEM of a 'typical' *Rosa corymbifera* 'Laxa' seed.

As with the light microscopy (plate 3.6), the suture can be seen running along the top of the seed (a to b). With this example of an untreated seed, the suture is firmly closed and the outer papery layer intact as far as c.

There is also a pair of hairs at the base of the seed (d). These are characteristic of *Rosa* species, and cause the irritation to the skin experienced when the seed is used as "itching powder".

NB The pitted area in the background is the conductive adhesive tape used to hold the seed in place on the metal stub in the microscope. This effect can be seen in the background of many of the scanning electron micrographs. In other micrographs the edge of the stub is also visible, obvious by its striated metal appearance and round edge.

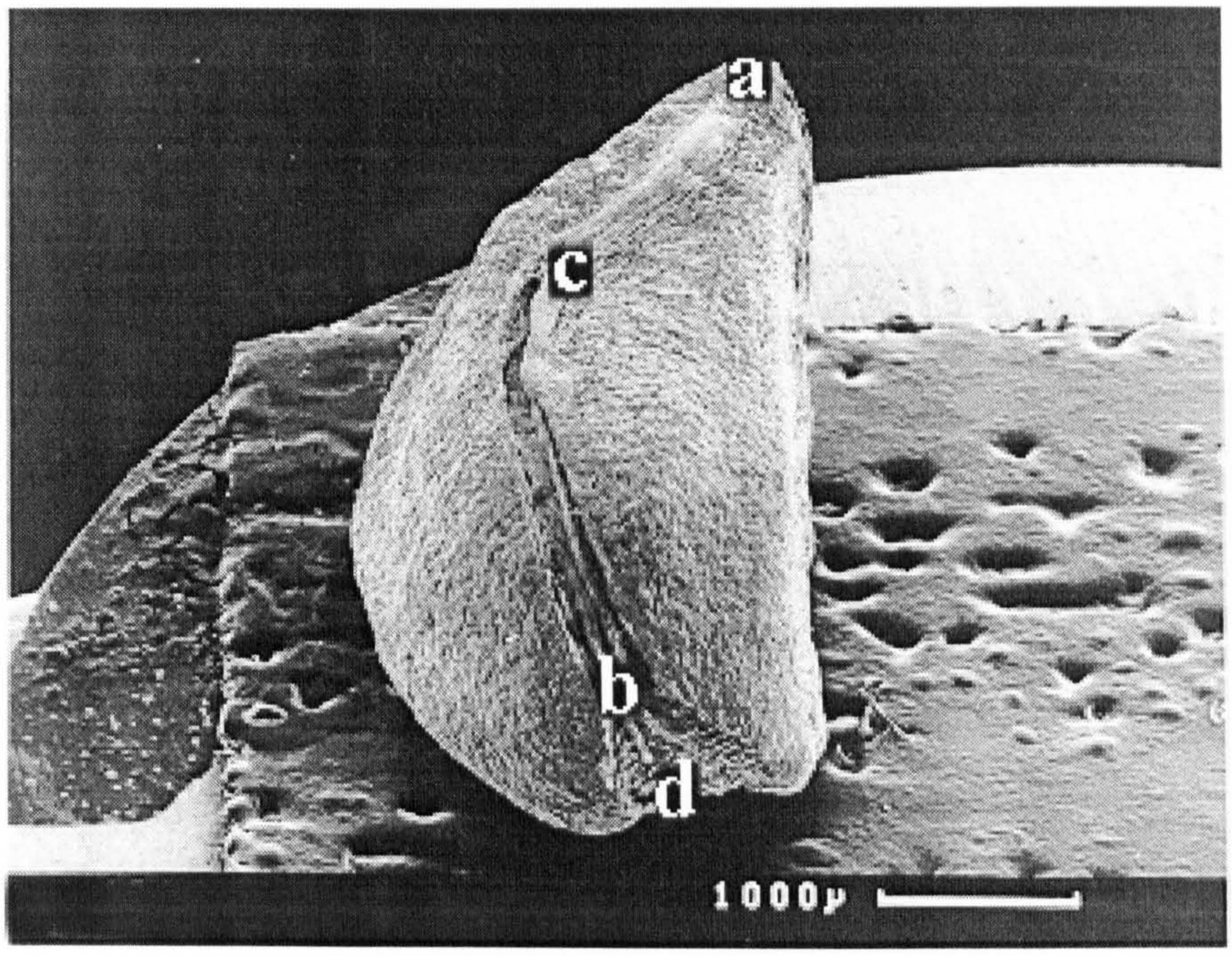


Plate 3.8 Another untreated representative of the same species.

The suture runs from a to b, the part between b and c shows where the papery outer layer has collapsed due to the drying (under vacuum) required for these samples. At the base of the seed is the hilum (d).

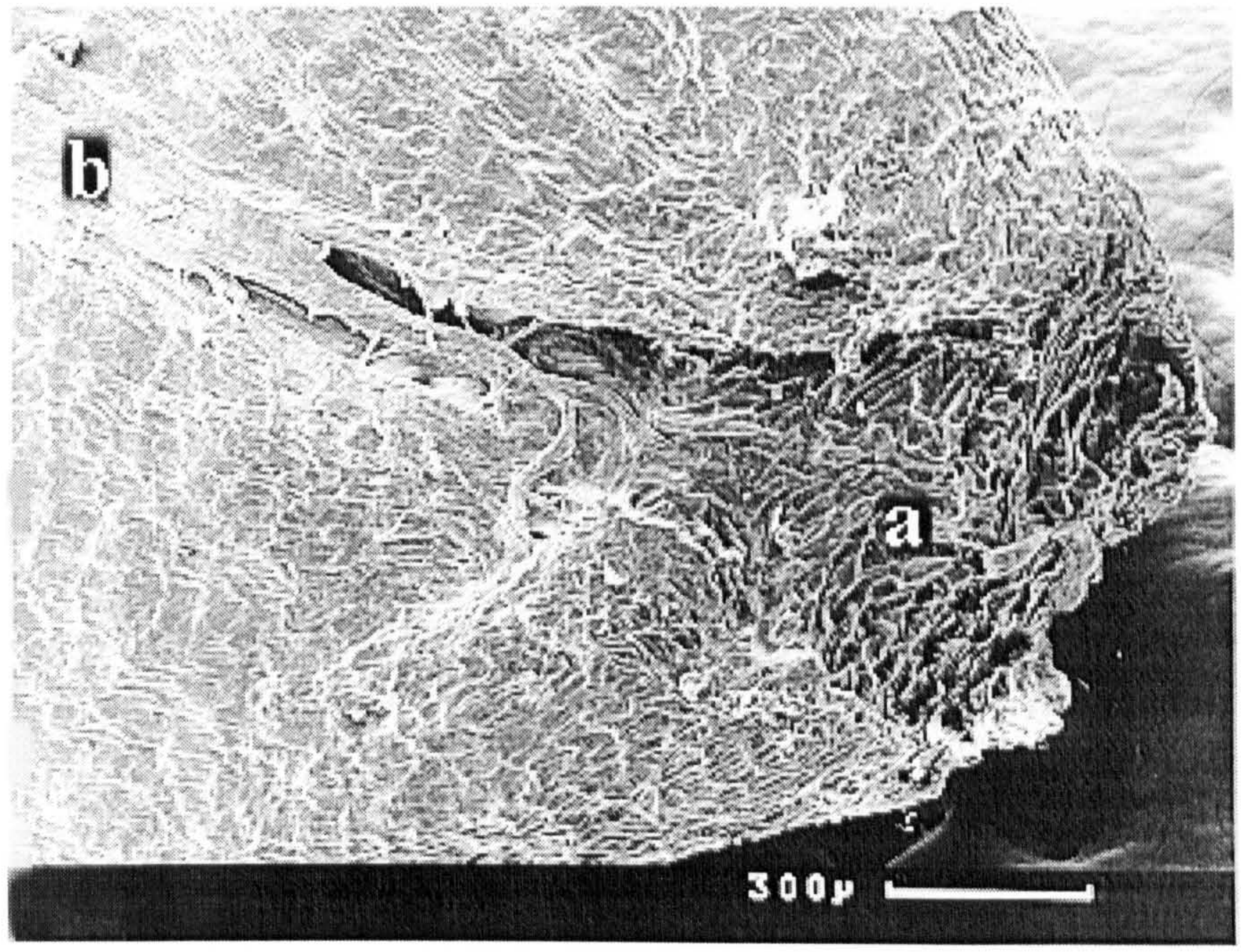


Plate 3.9 The hilum area magnified from plate 3.8.

In this plate the hilum is located to the right (a) and the suture runs away to the left (b). On an untreated *Rosa corymbifera* 'Laxa' seed the hilum appears to represent a more obvious weak point on the seed coat than the suture.

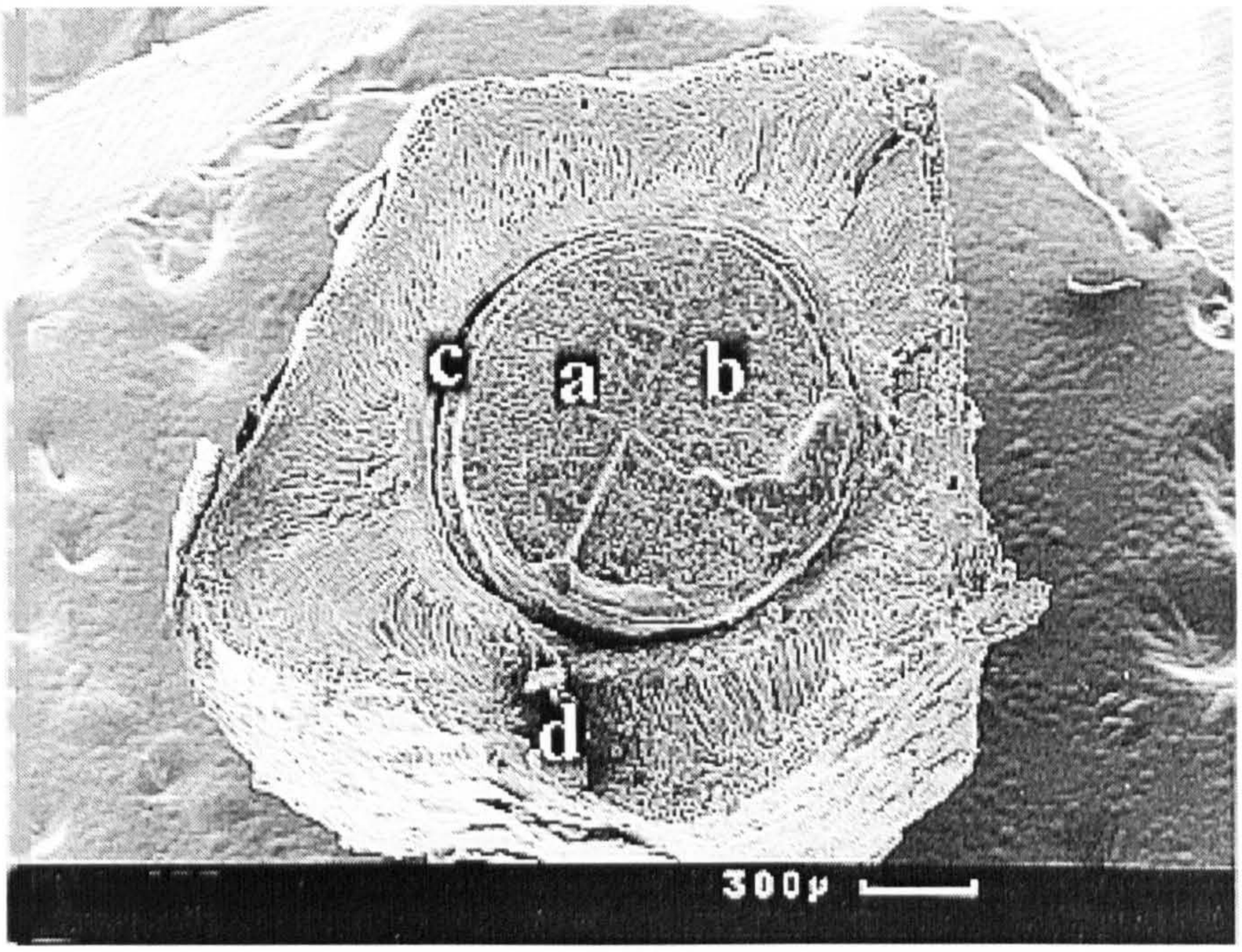


Plate 3.10 A cross section through an untreated *Rosa corymbifera* 'Laxa' seed.
The embryo is visible in the middle of the seed, with an obvious distinction between the two cotyledons (a and b). Moving outward from the embryo is the testa, which completely surrounds the embryo (c). The suture can be distinguished from the rest of the seed coat at the base of the plate (d).

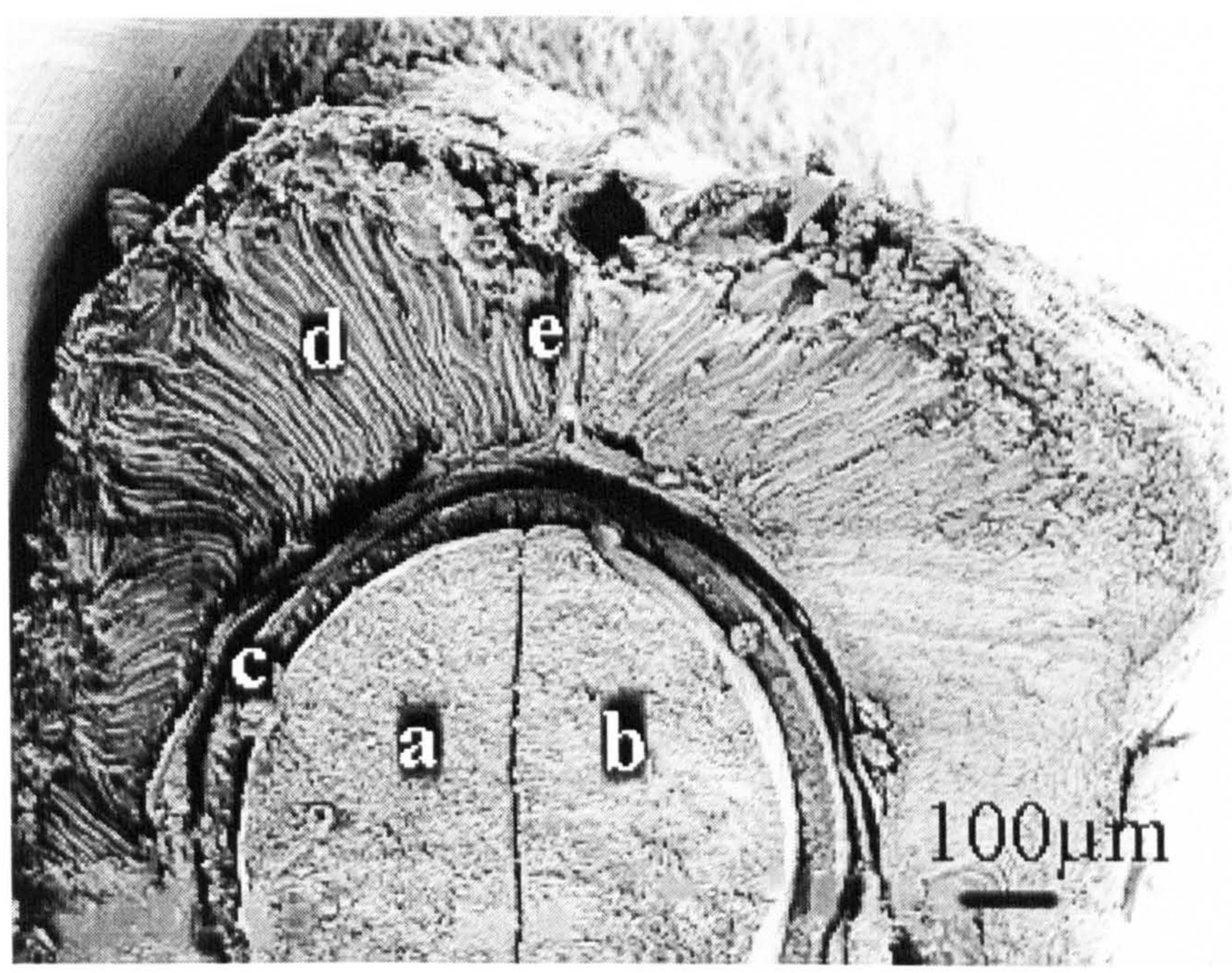


Plate 3.11 A closer view of a cross section taken from an untreated *Rosa corymbifera* 'Laxa' seed.
Again the two cotyledons constituting the embryo can be clearly seen (a and b). Immediately surrounding the embryo is the testa (c) and the seed coat (d). The suture in this seed lies at the twelve o'clock position (e).

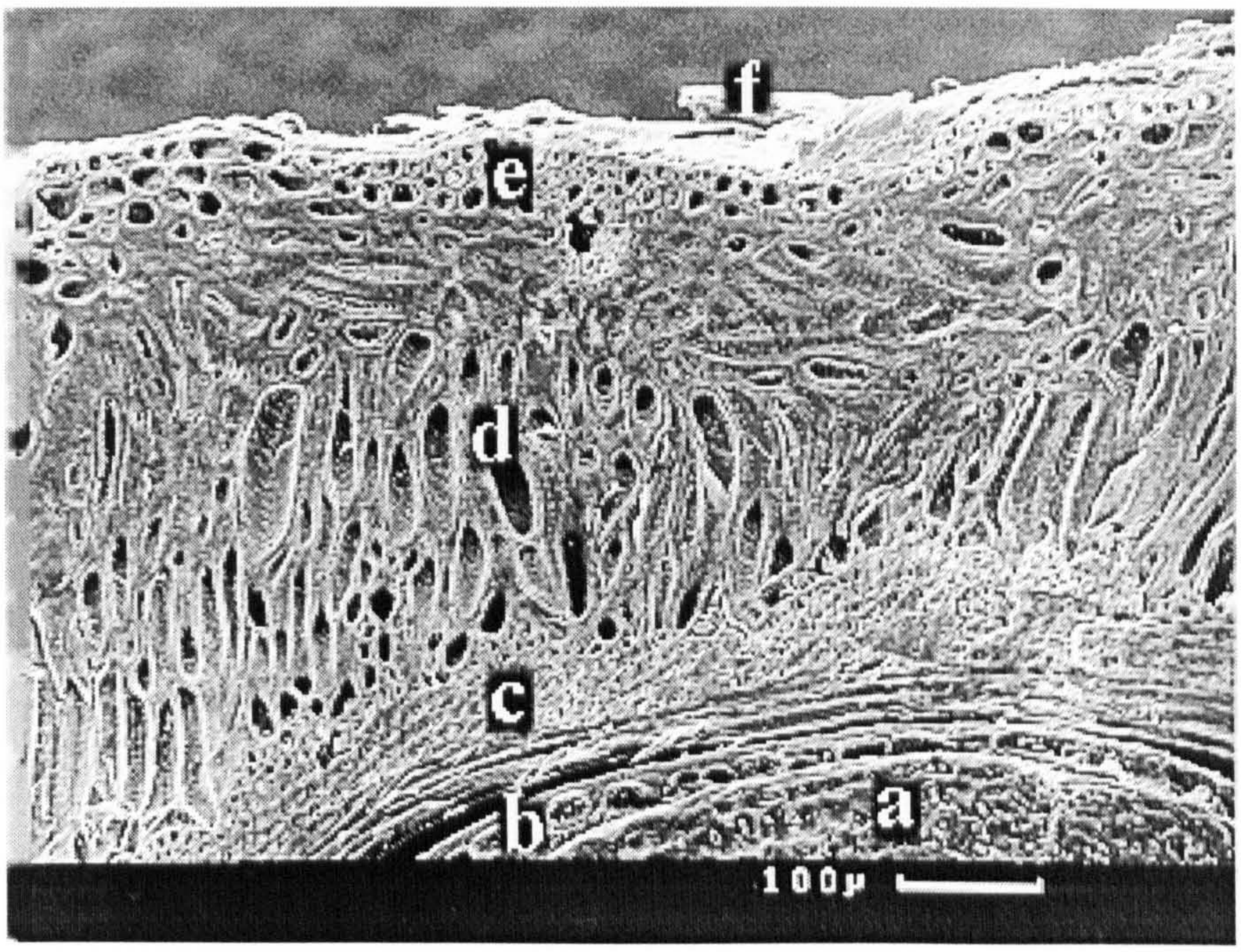


Plate 3.12 An enlarged area of the cross section of plate 3.10.

The orientation of the cells of the seed coat of *Rosa corymbifera* 'Laxa' is much clearer than in the last plates. To the base of the section lies the edge of the embryo (a) with the testa closely associated with it (b). The seed coat can then be divided into three discrete layers of cells, each lying perpendicular to each other. Moving from the bottom to top of the seed coat are the endocarp, mesocarp and exocarp (c, d and e respectively). To the outer extreme is the thin, papery covering (f).

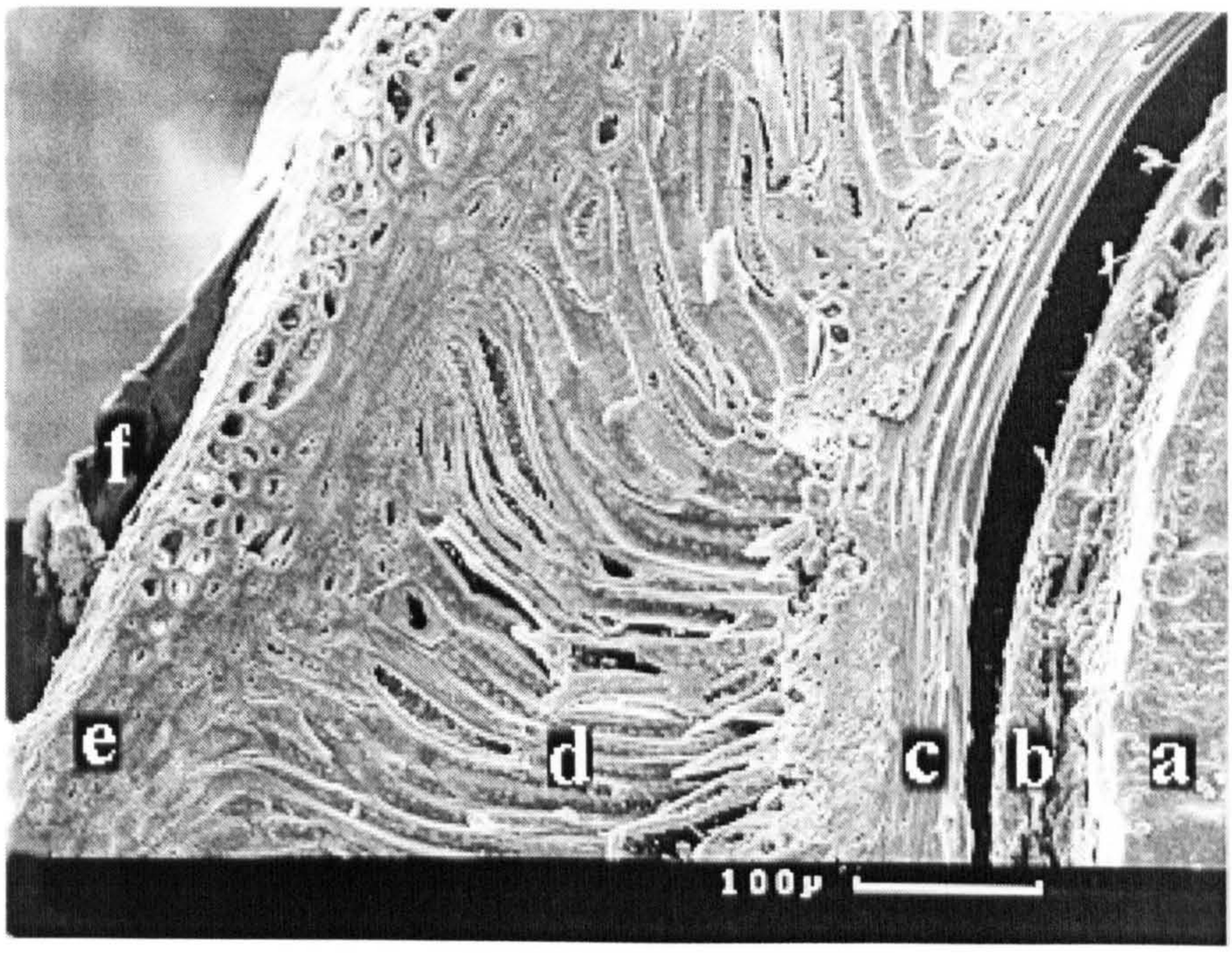


Plate 3.13 A second enlargement of plate 3.10 (taken from a different part of the seed). The different layers making up the seed coat are clearly seen, with the embryo to the far right (a) and the testa attached to it (b). The testa has pulled away from the endocarp (c), but can be seen as a discrete covering surrounding the embryo. The mesocarp and exocarp (d and e) lie to the left, with the papery layer (f) more obvious in this plate than the previous one.

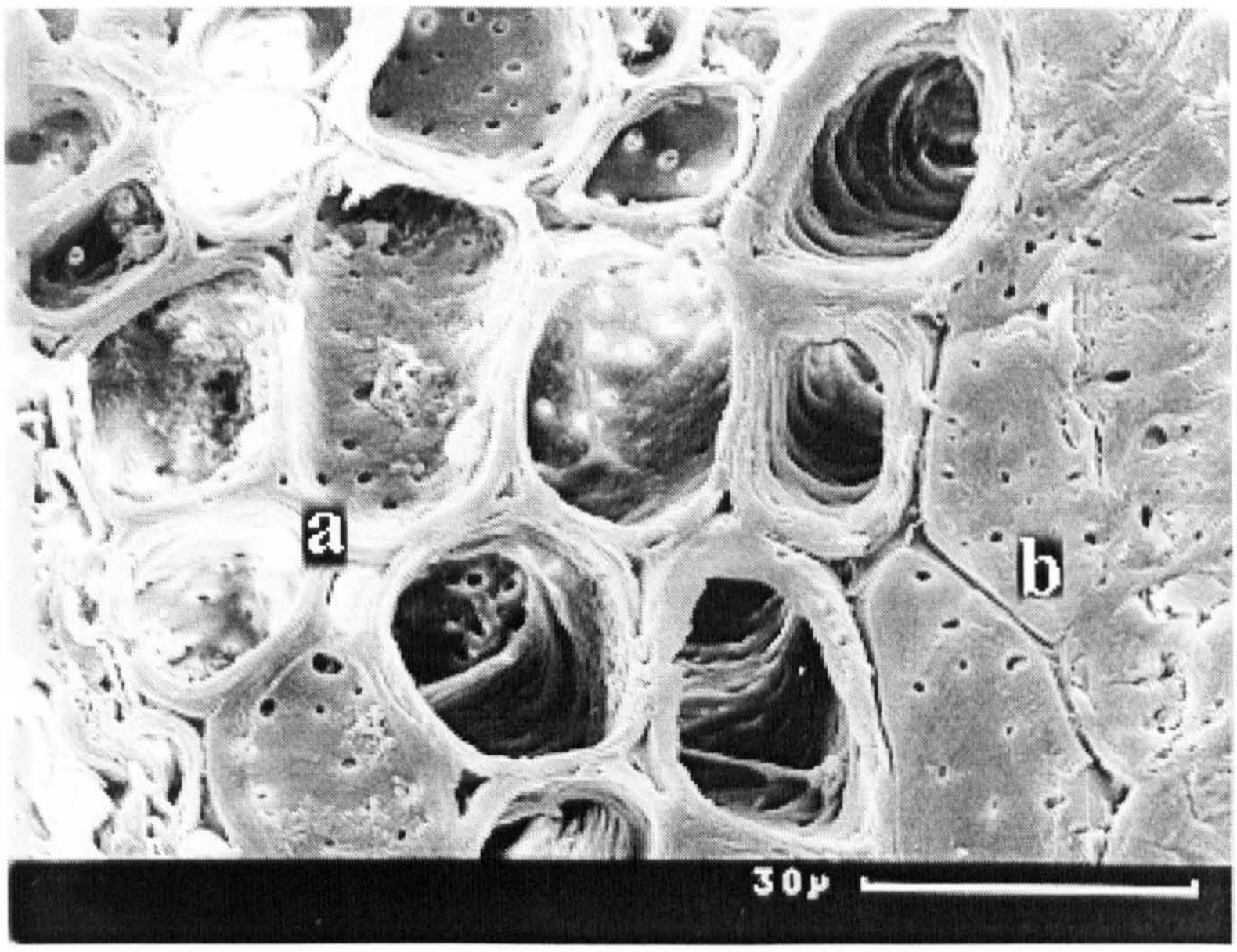


Plate 3.14 A higher magnification of the cross section in plate 3.10.
The structure of the sclerenchyma (of the exocarp (a) and mesocarp (b)) which forms the main body of the seed coat is shown clearly. This is tissue composed of sclerenchyma cells which are varied in form but typically are not elongated. They have thick lignified secondary walls with many pits. These pits arise from sclerification of parenchyma cells (cells with live nucleate protoplasts concerned with physiological activities).

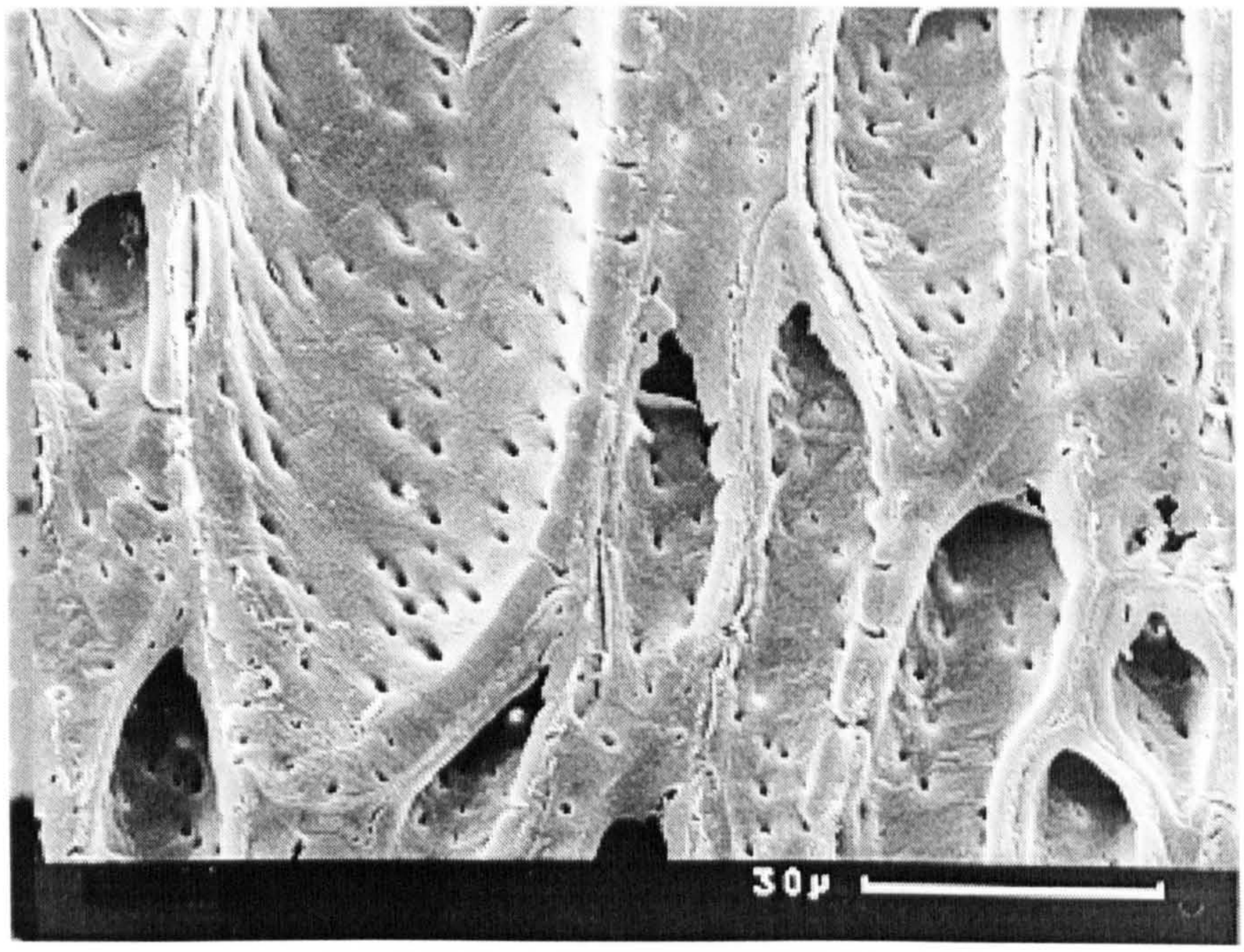


Plate 3.15 Highly magnified mesocarp from plate 3.10.
The small holes in the sclerenchyma cells can be seen scattered throughout the tissue. These are the pits which could also be seen in plate 3.14 and to a lesser degree in plates 3.12 and 3.13.

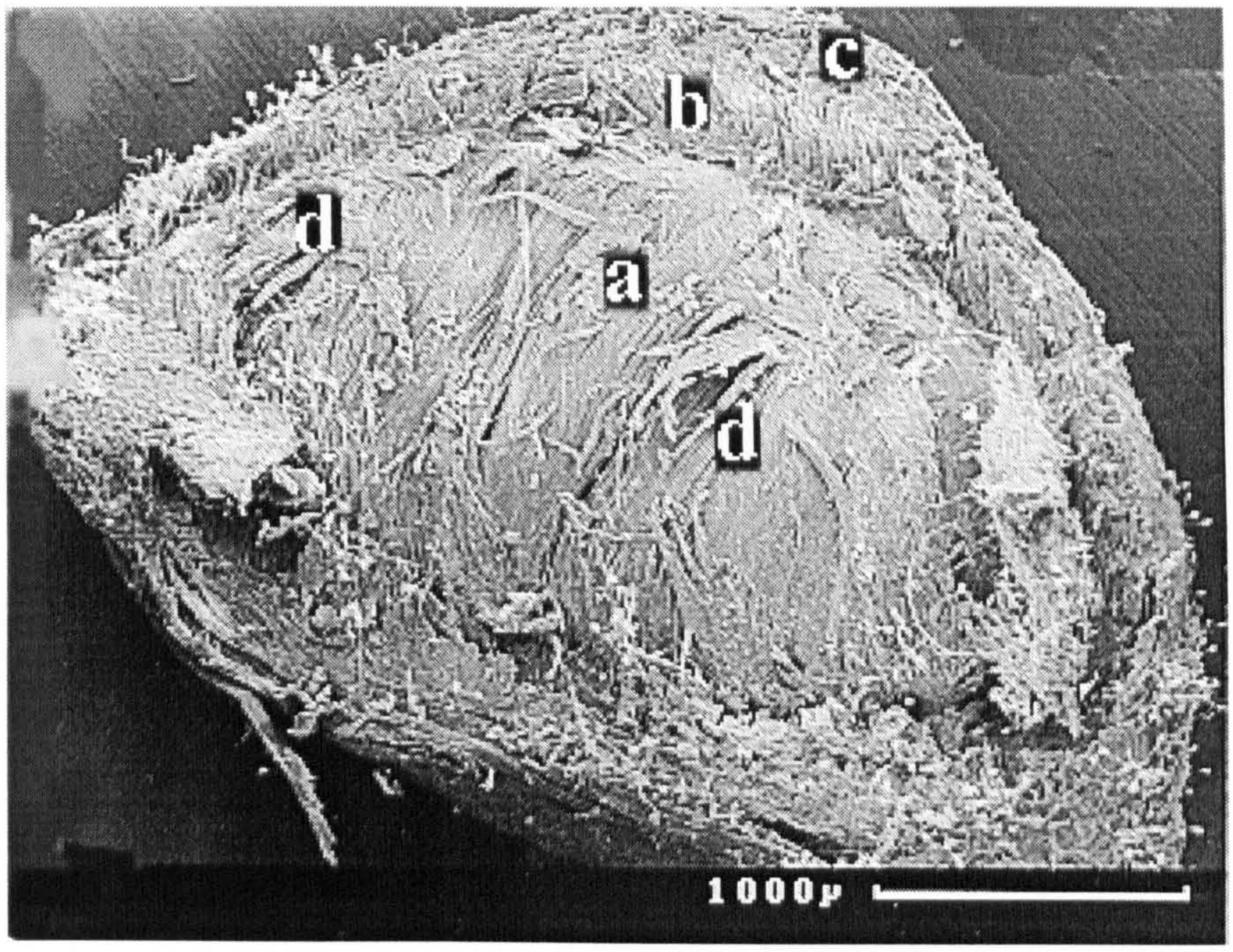


Plate 3.16 Longitudinal section of *Rosa corymbifera* 'Laxa' seed which has been pretreated with Garotta.
One half of the seed coat has been removed revealing the internal structures. Separation of the seed coat, however, was not complete. The endocarp (a) has remained intact around the embryo. The mesocarp (b) and exocarp (c) have split cleanly away. Parts of the endocarp layer have pulled away in several areas which clarifies the orientation of the cells (d).

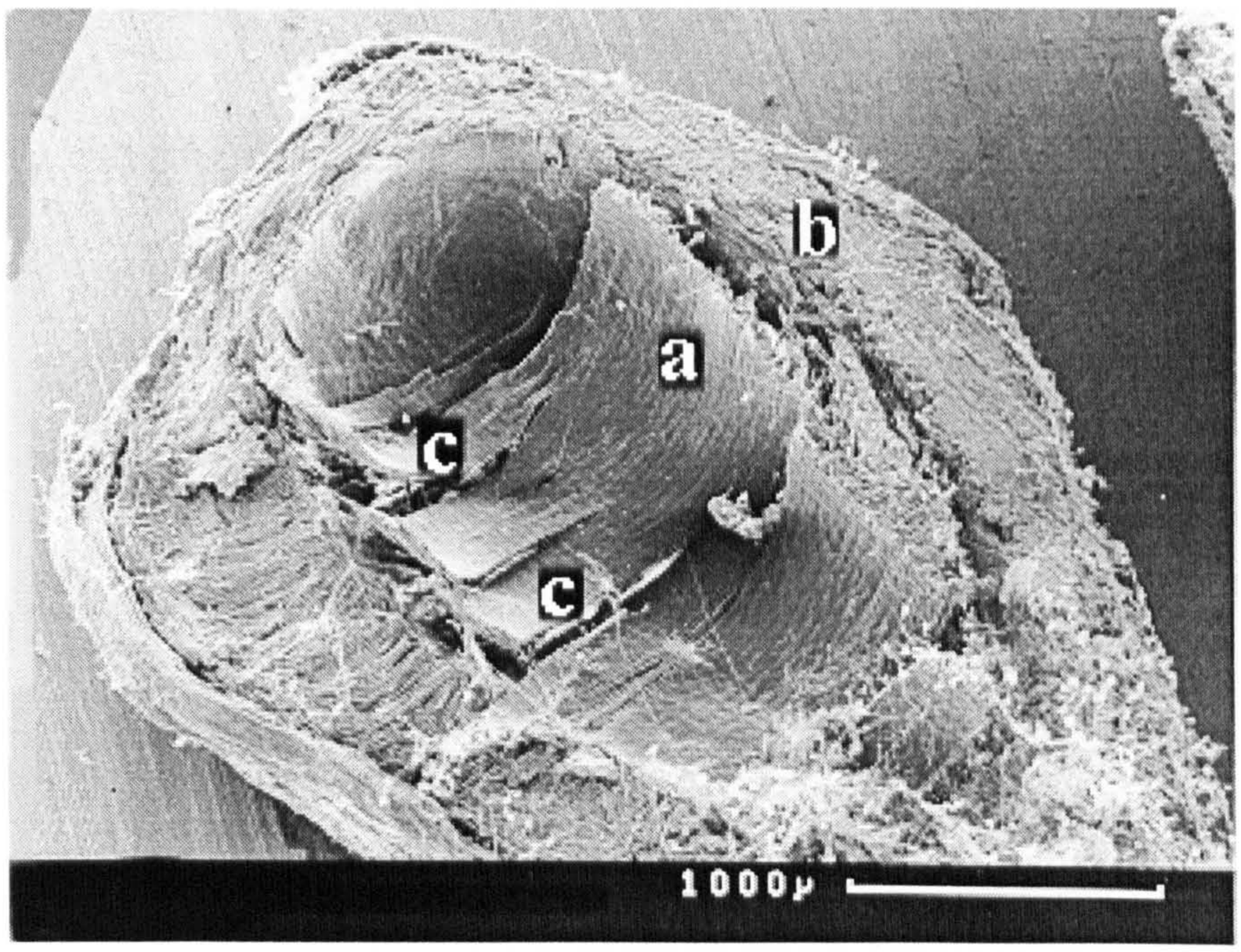


Plate 3.17 Longitudinal section of a Garotta pretreated *Rosa corymbifera* 'Laxa' seed.
In this example the embryo has been pulled out as the seed has split open. However, the majority of the endocarp from this half of the seed has remained. The endocarp layer (a) has separated from the mesocarp (b). The endocarp has torn in places (c) along the orientation of the cells within that layer. This clarifies their orientation.

Plate 3.18 Untreated seed of *Rosa corymbifera* 'Laxa'.

The cut open seed is light brown both on the internal and external surfaces.

Plate 3.19 Lignin staining of *Rosa corymbifera* 'Laxa' seeds.

The areas stained red indicate lignin. Lignin is a major component of the seed coat, mainly found in the sclerenchyma.

Plate 3.20 Cellulose staining of *Rosa corymbifera* 'Laxa' after the first step in the staining procedure.

The blue colouration is the initial staining of all the polysaccharide material.

Plate 3.21 Cellulose staining of *Rosa corymbifera* 'Laxa' after the second step in the staining procedure.

The swelling of the cellulose fibres is visible and stains as a yellow/brown colour.

3.1.3 Discussion

To date the anatomy of *Rosa corymbifera* 'Laxa' was poorly reported in the literature. However, detailed study of the seed, culminating in the plates in the previous section (3.1.2), have resulted in a detailed knowledge of the structure being obtained.

Plates 3.1, 3.2 and 3.3 set the scene for the origin of *Rosa corymbifera* 'Laxa' in relation to the mother plant and how the seed forms. The subsequent three plates used normal colour photography to introduce the size, colour and shape aspects of *Rosa corymbifera* 'Laxa' seed. The presence of a suture - the area of structural weakness, was also clearly seen in these results.

Scanning electron microscopy was then used to show more detail of the structure of *Rosa corymbifera* 'Laxa' seed. Even at magnifications equivalent to the conventional microscopy, detail was clearer and better defined due to the high resolution and contrast of scanning electron microscopy. Thus the first two electron micrographs (plates 3.7 and 3.8) were of seed at very low magnification. This allowed easy transition and comparison between the conventional colour photography and the high resolution black and white scanning electron microscopy.

Plates 3.9 to 3.17 revealed the finer detail required for completing the structural picture for *Rosa corymbifera* 'Laxa' seed. Two areas of structural weakness were found, the suture and the hilum. The hilum and suture represent the only weaknesses in the hard seed coat. These areas would be the most vunerable to microbial attack or water penetration. This could be of particular importance with regard to germination, as the seed coat must be breached before this event can proceed.

Once good quality sections were made of the seed, the internal structure could be examined. Distinct layers were found making up the pericarp (plates 3.11, 3.12 and 3.13), the fundamental difference between them being the orientation of the cells. In the three layers of cells making up the pericarp, boundaries are clear between them, and when studied the cells were found to be orientated perpendicular to each other. In effect each layer could be assigned a unique plane within the pericarp. This layering could be to strengthen the coat and help restrict water uptake. The advantage of a strong seed coat

would be to protect the embryo, and any restriction of water uptake would protect the embryo from varying states of hydration/dehydration.

The plates in section 3.1.2 show the discrete layers making up the seed coat of *Rosa corymbifera* 'Laxa'. The orientation of the cells was also evident. These results have been used to produce the line drawing below (figure 3.1), showing clearly the structural composition of the seed.

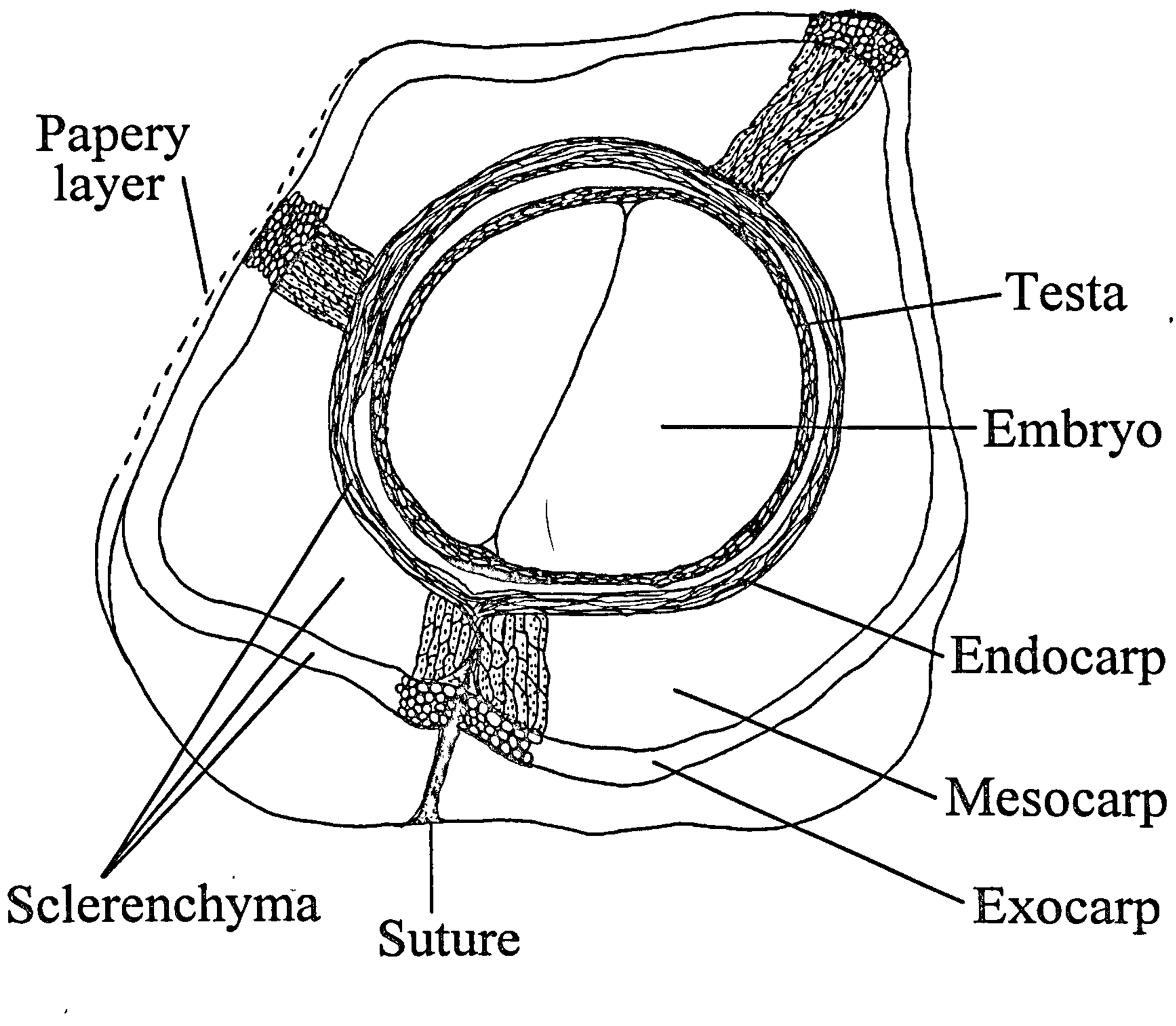


Figure 3.1 Composite drawing of the cross section of *Rosa corymbifera* 'Laxa', produced from the results in section 3.1.2 (plates 3.10 through to 3.17).

Similar studies on other species of Rose and other hard coated *Rosaceous* species would be very useful to look for commonalities within seed coat structures. This could help in deciding how to pretreat similar species to *Rosa corymbifera* 'Laxa'.

3.2 Colour and structural changes of seeds prior to germination

Objective - to identify any physical differences between commercial and Garotta pretreated seeds of *Rosa corymbifera* 'Laxa' following the pretreatments.

3.2.1 Materials and Methods

Light microscopy and scanning electron microscopy were used to record any physical changes occuring to or on the seeds. The materials and methods for this section are therefore as described in section 3.1.1. Samples of seed were taken at the end of the 24 week commercial (detailed in section 2.1.1) and 24 week Garotta pretreatments (detailed in section 2.3.1).

3.2.2 Results

During the pretreatment of *Rosa corymbifera* 'Laxa', the seed undergoes certain discernible changes before germination. The most obvious difference is a colour change of the seed between the commercial and Garotta treated seed. Plate 3.22 clearly shows this difference.

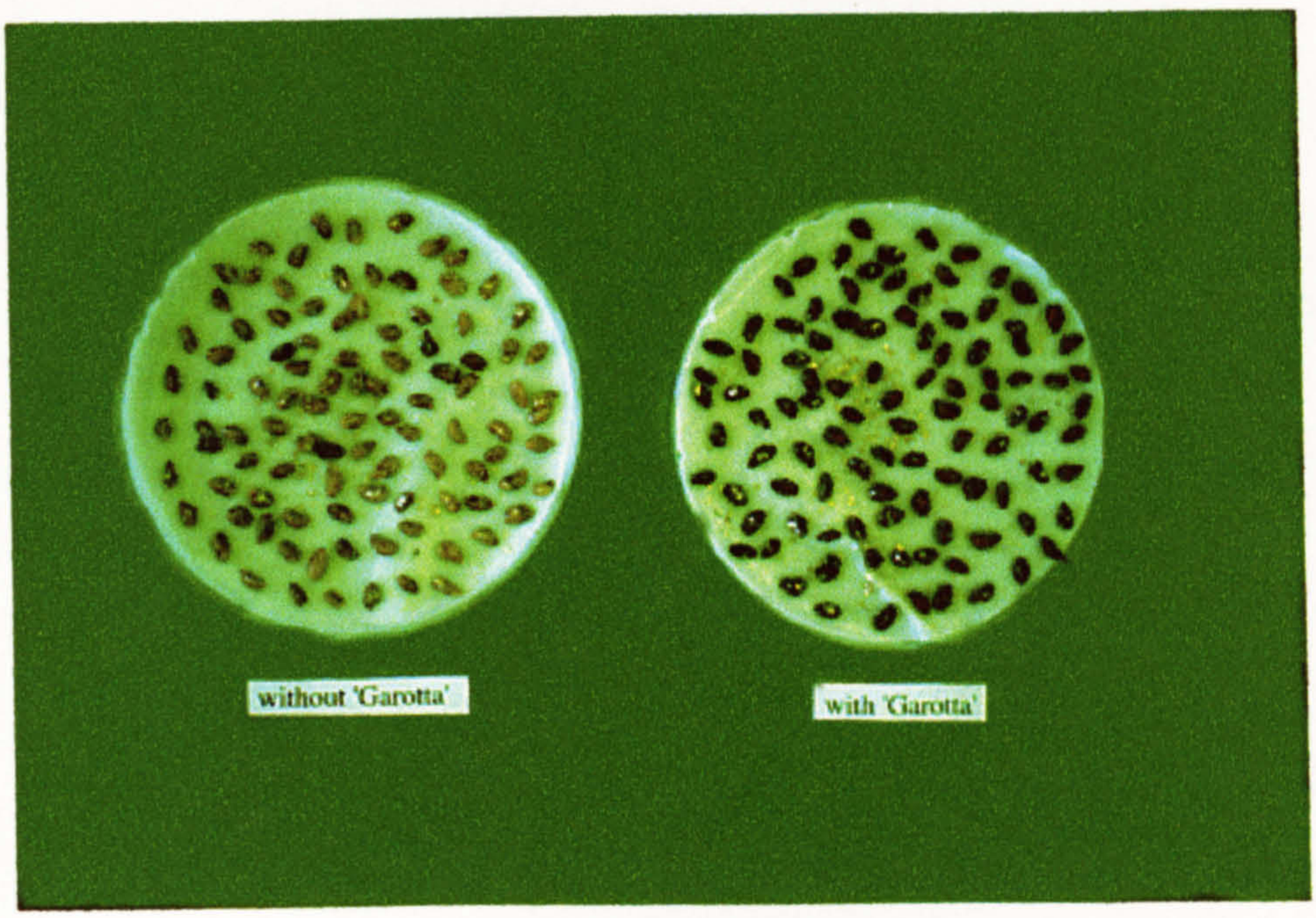


Plate 3.22 Pretreated seed of *Rosa corymbifera* 'Laxa'.
The commercially pretreated seed (to the left) remains a light brown, not differing too much from the colour of untreated seed. However, the Garotta treated seed turns a very marked dark brown.

The colour change is first noticed after only 2 - 3 weeks into the warm period of pretreatment. Whilst the Garotta treated seed rapidly turns dark brown, commercially treated seed remains unchanged, even after 12 weeks warm incubation. In one replicate of one experiment, some commercially pretreated seed did darken slightly, however it remained much lighter than the Garotta equivalents.

Before *Rosa corymbifera* 'Laxa' seed will germinate the seed coat must be sufficiently weakened to allow the embryo to emerge. This mechanical constraint is one of the typical dormancy traits of hard coated seed (see section 1.2). The weakening occurs along the suture, described in section 3.1.2. Following the commercial pretreatment very few (less than 5%) of the seeds have split, whereas following the Garotta pretreatment over 90% had split (see appendix 3.3 for figures). Plates 3.23 and 3.24 (overleaf) show more clearly this effect, and also the colour difference previously described. Plates 3.25 - 3.30 show clearly the splitting of Garotta pretreated seed and the unsplit commercially pretreated seed.

Plate 3.23 *Rosa corymbifera* 'Laxa' seed after the commercial pretreatment (Mag^{n} x3). The seed is light brown in colour with no splitting of the suture.

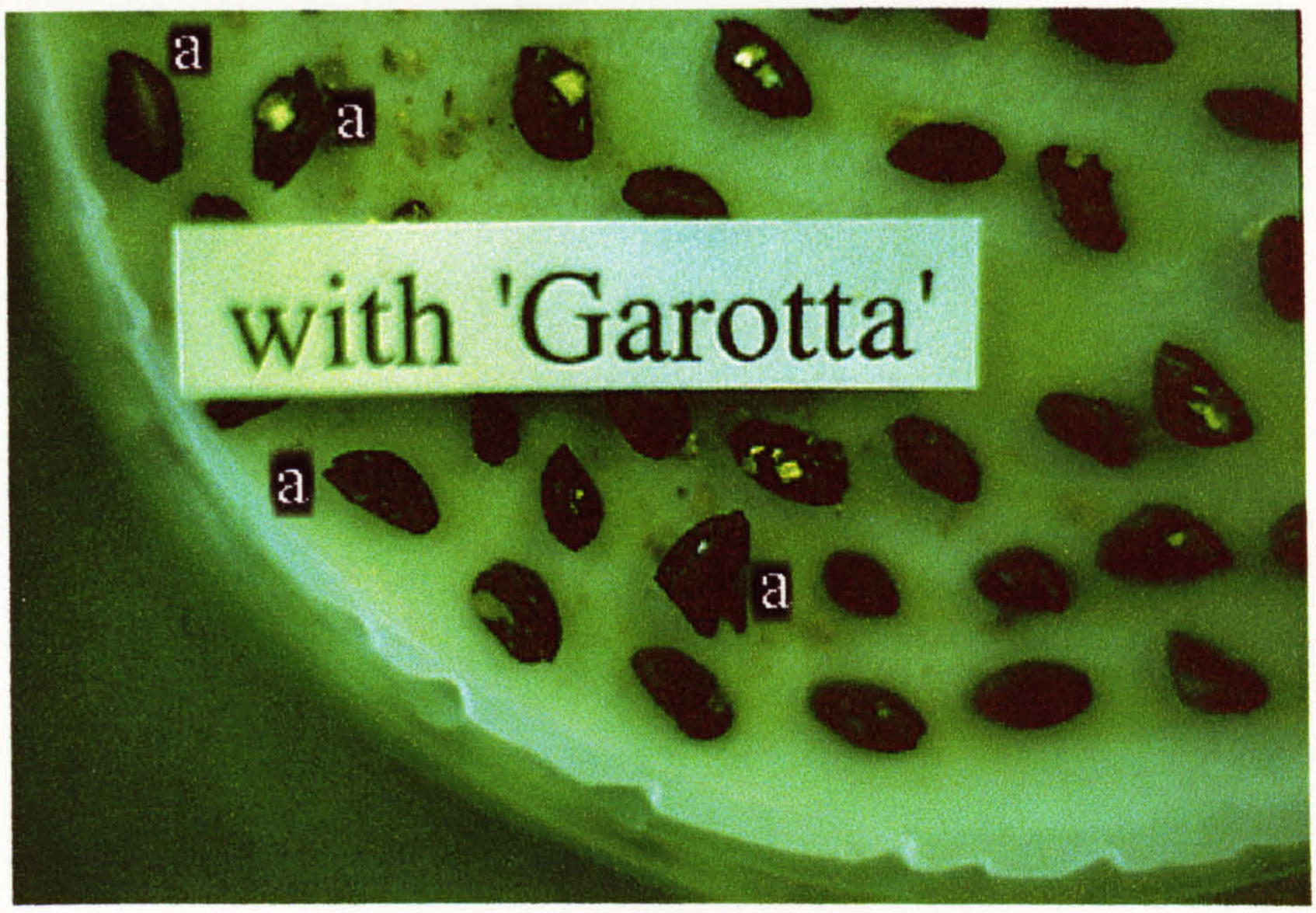


Plate 3.24 *Rosa corymbifera* 'Laxa' seed after the Garotta pretreatment (Mag^{n} x3). The seed is dark brown and most are split along the suture. The seeds which have shown the most obvious splitting are labelled 'a'. However, all the seeds in this plate have actually split.

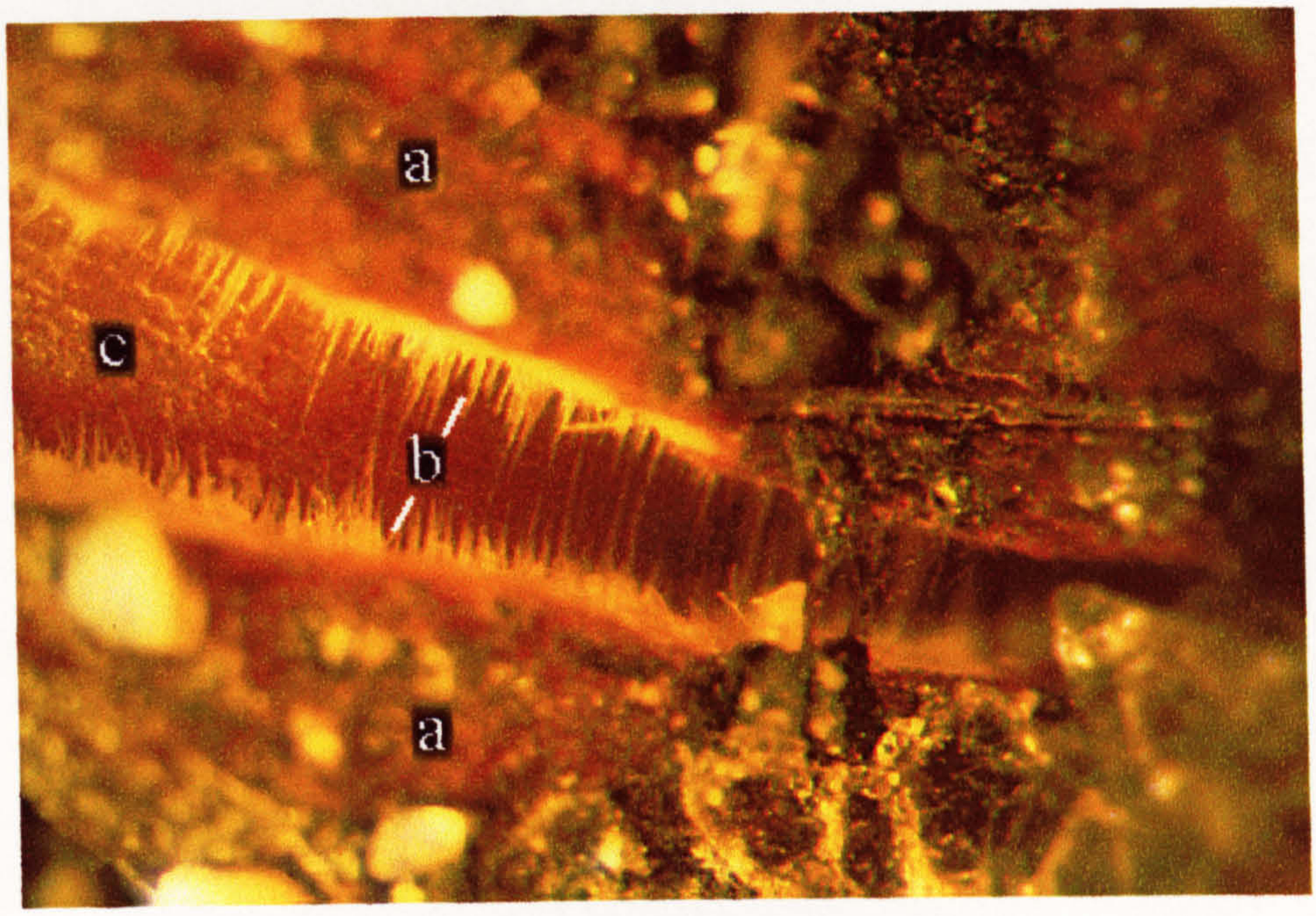


Plate 3.25 A close up of a split seed of *Rosa corymbifera* 'Laxa' following pretreatment with Garotta (Mag^n x125).

This plate shows the final pulling apart of the of the seed coat (a) which occurs during the warm period of incubation. The 'ripping' effect (b) seen in this plate is due to the last sclerenchyma fibres of the seed coat having pulled apart. The embryo is also clearly seen (c).

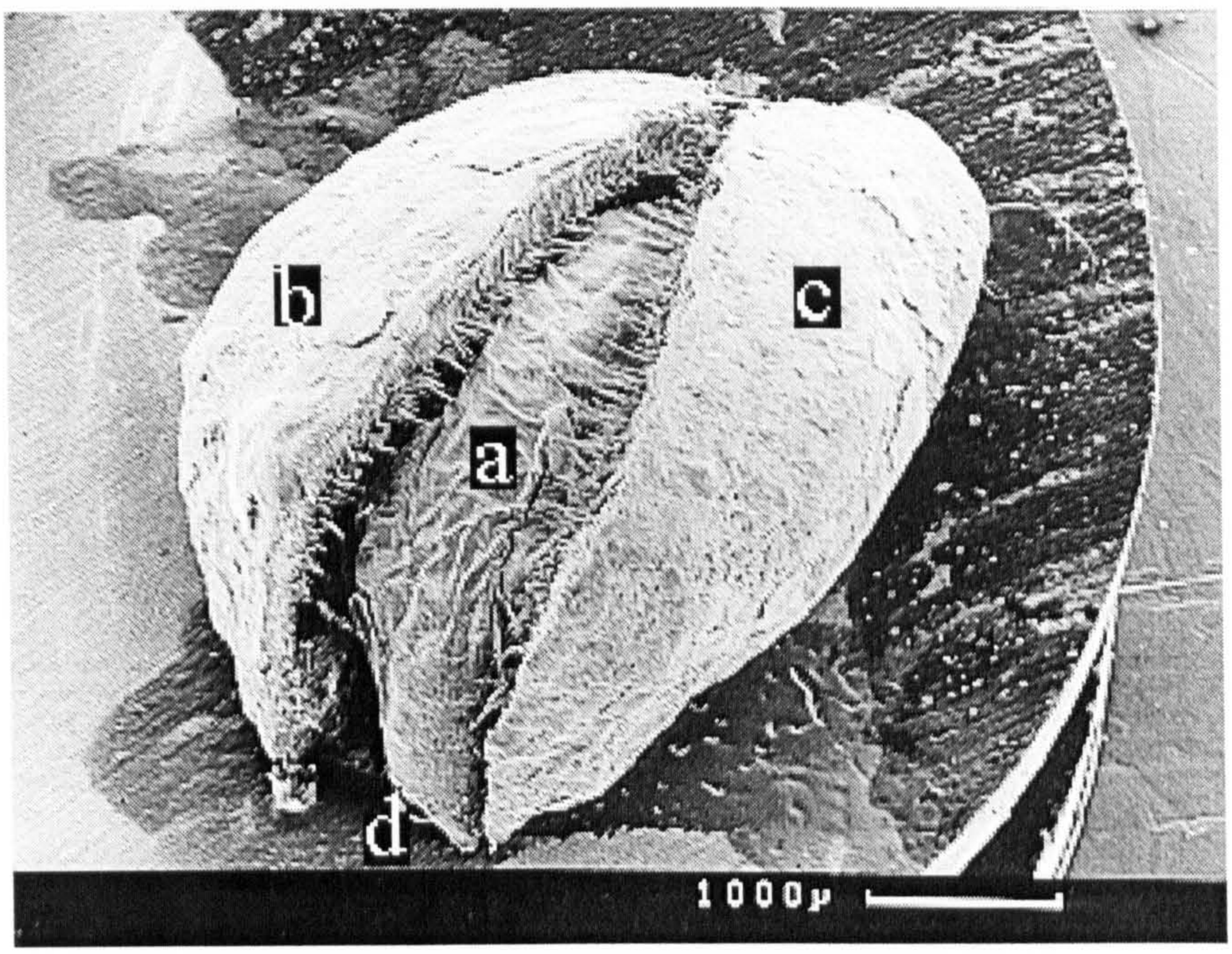


Plate 3.26 An SEM of *Rosa corymbifera* 'Laxa' following pretreatment with Garotta. This seed was at the same stage as those described previously in plates 3.24 and 3.25. This plate shows a Garotta treated seed split along the entire length of the suture. The embryo (a) is visible in the centre, which shows slight signs of shrivelling. This was caused by the drying of the seed prior to viewing under the microscope. The two halves of the seed coat have pulled away from each other (b and c). The early stage of the radicle (embryonic root) can be seen in at the base of the seed (d).

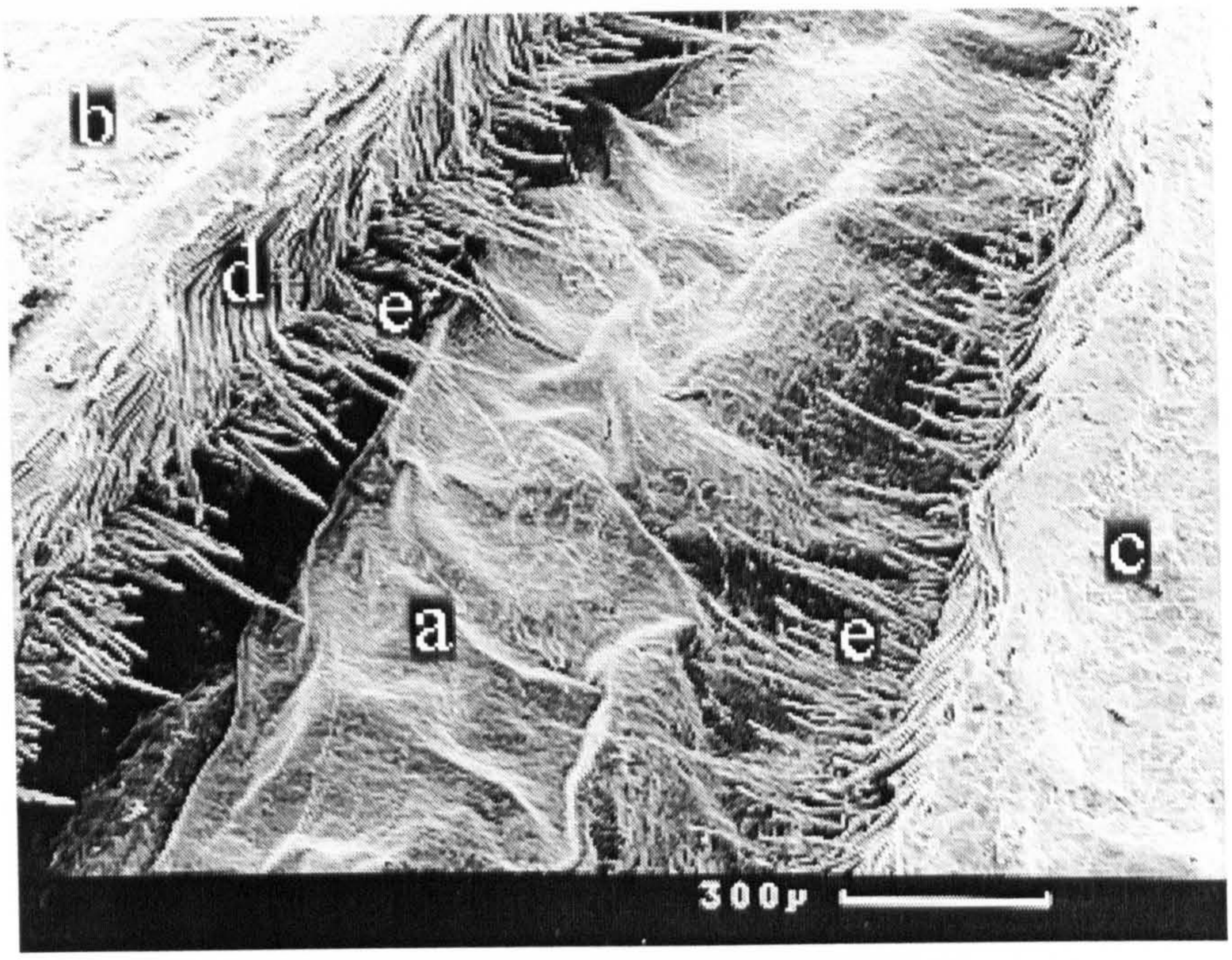


Plate 3.27 A magnified view of plate 3.26.

The seed has split open revealing the embryo (a). The two halves of the seed coat (b and c) are visible to the left and right of the plate. A close look where the suture has separated shows which particular fibres were last to pull apart. The fibres of the mesocarp had already separated cleanly (d), however, those on the inner side, the endocarp (e), have not - they have been pulled apart under some force (probably due to the embryo swelling sufficiently to overcome this last mechanical constraint).

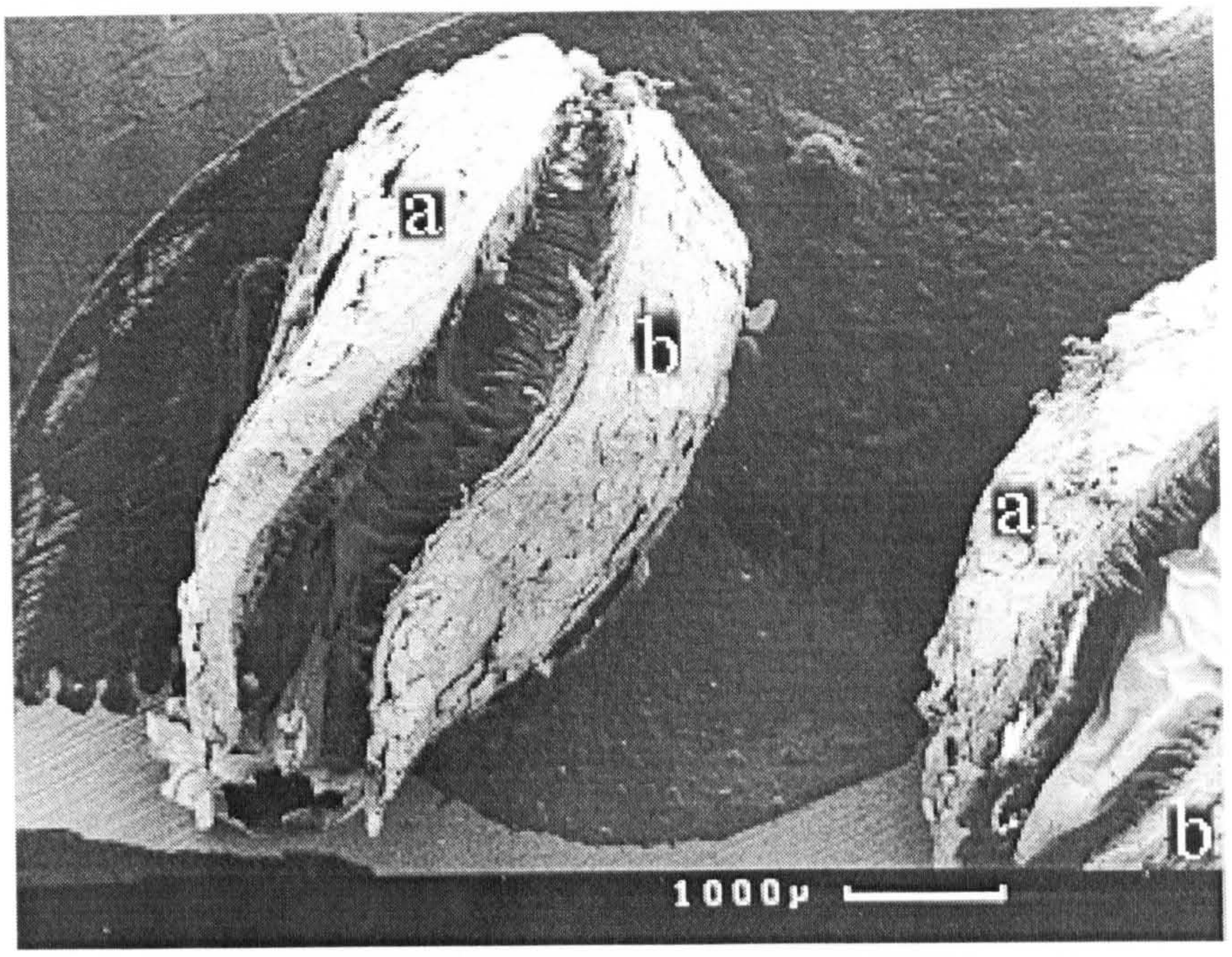


Plate 3.28 Two further examples of split seed taken from the Garotta pretreatment. The parting of the two halves of the seed coat is very obvious (a and b). This splitting was easily recognised by the naked eye.

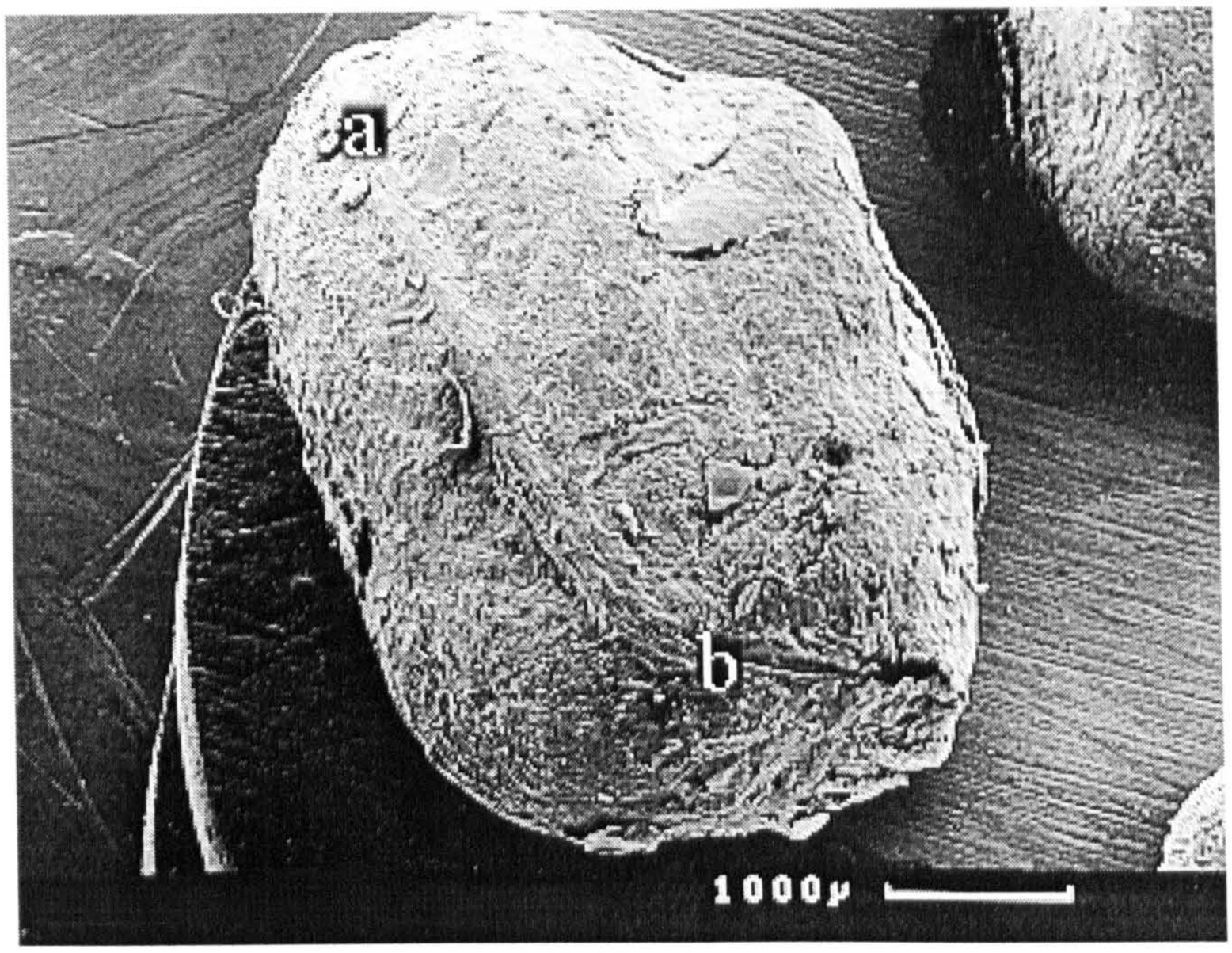


Plate 3.29 A typical *Rosa corymbifera* 'Laxa' seed taken at the end of the commercial pretreatment.

The suture is clearly visible along the seed coat (a to b). It usually remains firmly closed in commercially pretreated seed, as seen in this example.

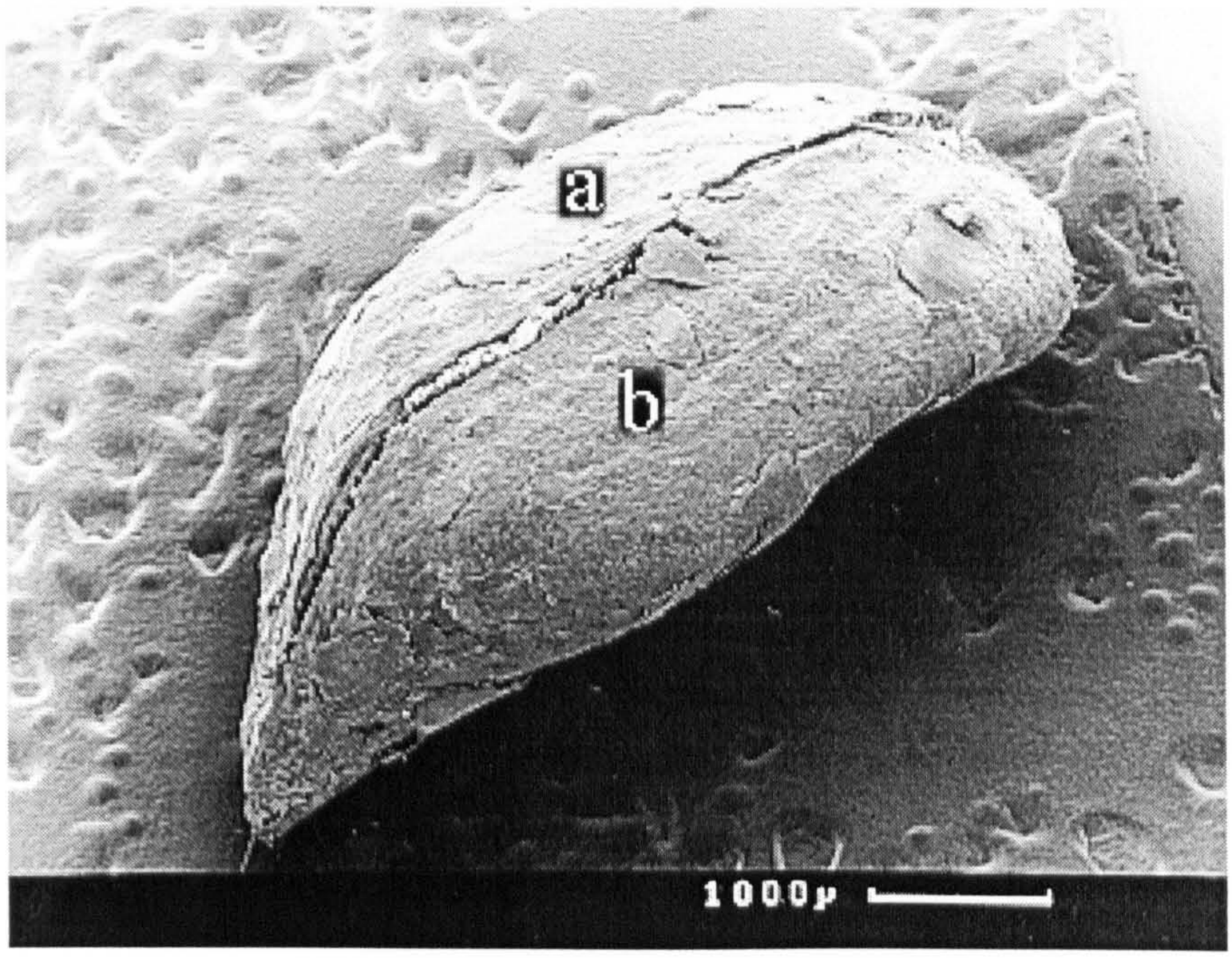


Plate 3.30 An example of a split seed from the commercial pretreatment.

Occasionally some commercially pretreated seeds of *Rosa corymbifera* 'Laxa' did split, although this was only found in approximately 5% of cases (appendix 3.3). Even when they did show signs of splitting, the effect was not as obvious as that seen with the Garotta pretreated seed. Not all the layers of the seed coat have separated and hence only slight splitting has resulted (a to b).

3.2.3 Discussion

During the warm period of the pretreatments of *Rosa corymbifera* 'Laxa' certain obvious physical changes occurred to the seed. After only 2 to 3 weeks the seed pretreated with Garotta had started darkening colour to dark brown, whilst that of the commercially treated seed remained unchanged relative to untreated seed (plates 3.22, 3.23 and 3.24). The likely cause of this is the effect of microbes on the seed coat. Certain groups of fungi, known as the brown rot fungi, are able to preferentially degrade cellulose and hemicellulose (Schlegel, 1993) leaving brown residues of phenyl compounds. However, these fungi tend to decompose wood (i.e. the seed coat) very slowly, hence a brown colour would become obvious but actual physical damage could take many months (Campbell, 1985).

By the end of the warm period over 95% of the Garotta treated seeds had split open along the suture (appendix 3.3). The seed coat had also softened and could be easily cut. The commercially treated seed on the other hand did not markedly change colour, and remained a light brown (plate 3.23), similar to untreated seed. The seed did not show signs of splitting (except in a few cases, in the order of 5%) and was as hard as it was at setup. Only a strong blade could breach the seed coat.

Light and scanning electron microscopy clearly showed the splitting of the seed, and which part of the seed coat was last to separate (plates 3.25 to 3.28) in the Garotta treated seed. Plates 3.23, 3.29 and 3.30 showed that *Rosa corymbifera* 'Laxa' seed which underwent a commercial treatment did not split open. Thus the physical differences between commercial and Garotta treated seed were markedly different.

3.3 Microbially loaded seed

If microbes enter the pretreatment of *Rosa corymbifera* 'Laxa' on the seed, then it would be reasonable to assume that examination of seed would reveal their presence.

Objective - to use scanning electron microscopy to ascertain the presence of microorganisms on *Rosa corymbifera* 'Laxa' seed.

3.3.1 Materials and Methods

Air dried *Rosa corymbifera* 'Laxa' seed was examined using scanning electron microscopy as described in section 3.1.1. To further investigate any microbial presence on the seed entering the pretreatment mix, untreated samples (i.e. only extracted from the hip, washed and air dried) were sealed in sterile eppendorfs with a drop of sterile tap water for 48 hours at 25°C. This provided an atmosphere conducive to microbial growth whilst eliminating external contaminants.

3.3.2 Results

Seed which was examined when air dried following extraction from the hips had some microbial loading, although this was restricted to the tip of the seed. There were no detectable signs on the rest of the seed. Plate 3.31, 3.32 and 3.33 show the results of these findings.

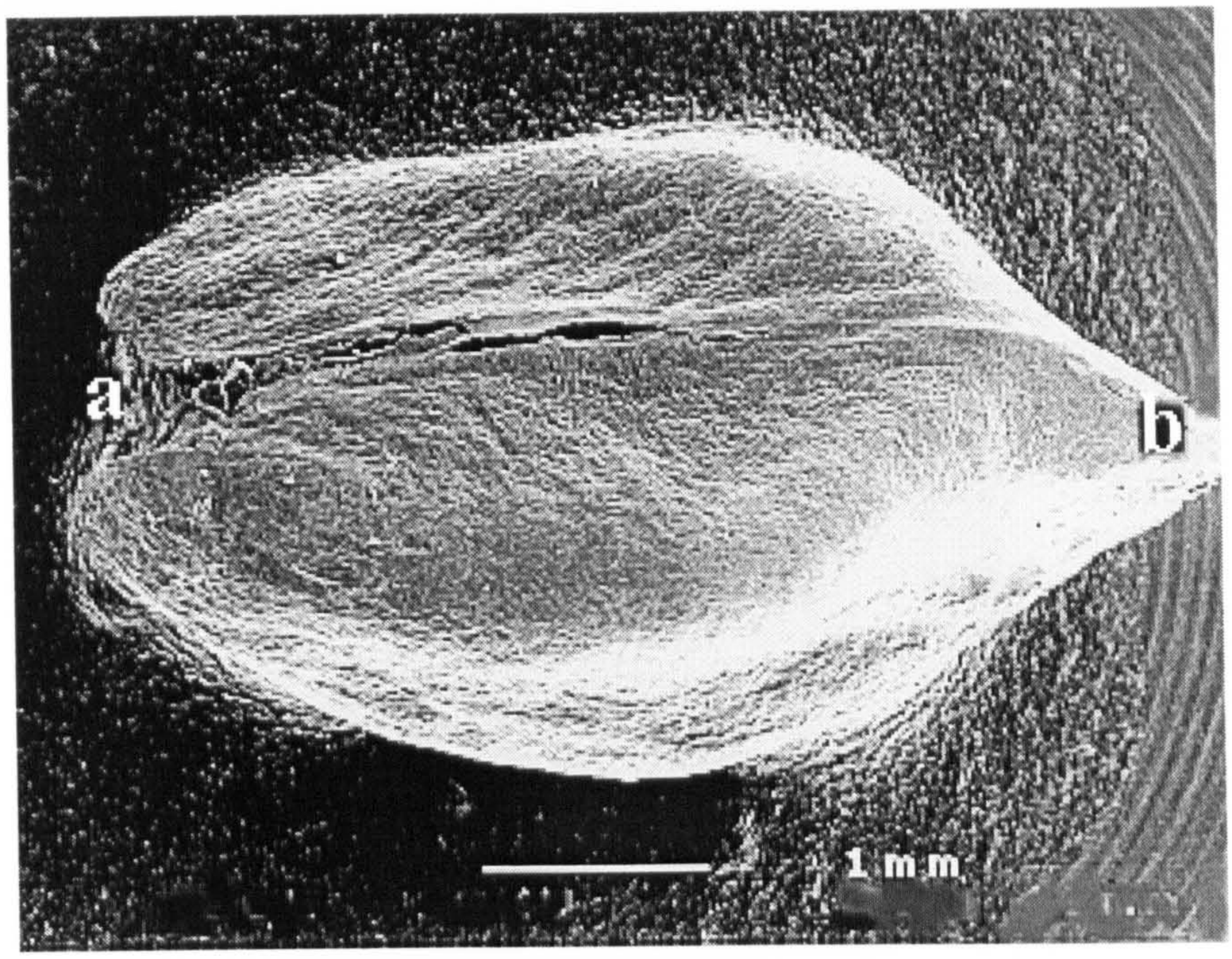


Plate 3.31 An unpretreated seed of *Rosa corymbifera* 'Laxa'.

At this magnification there were no detectable signs of microbial loading.

The hilum is located to the left of the plate (a) and the suture runs from the hilum to the tip of the seed (b). The vacuum drying of the this seed has caused the papery layer to collapse, showing the suture more clearly than would normally be expected.

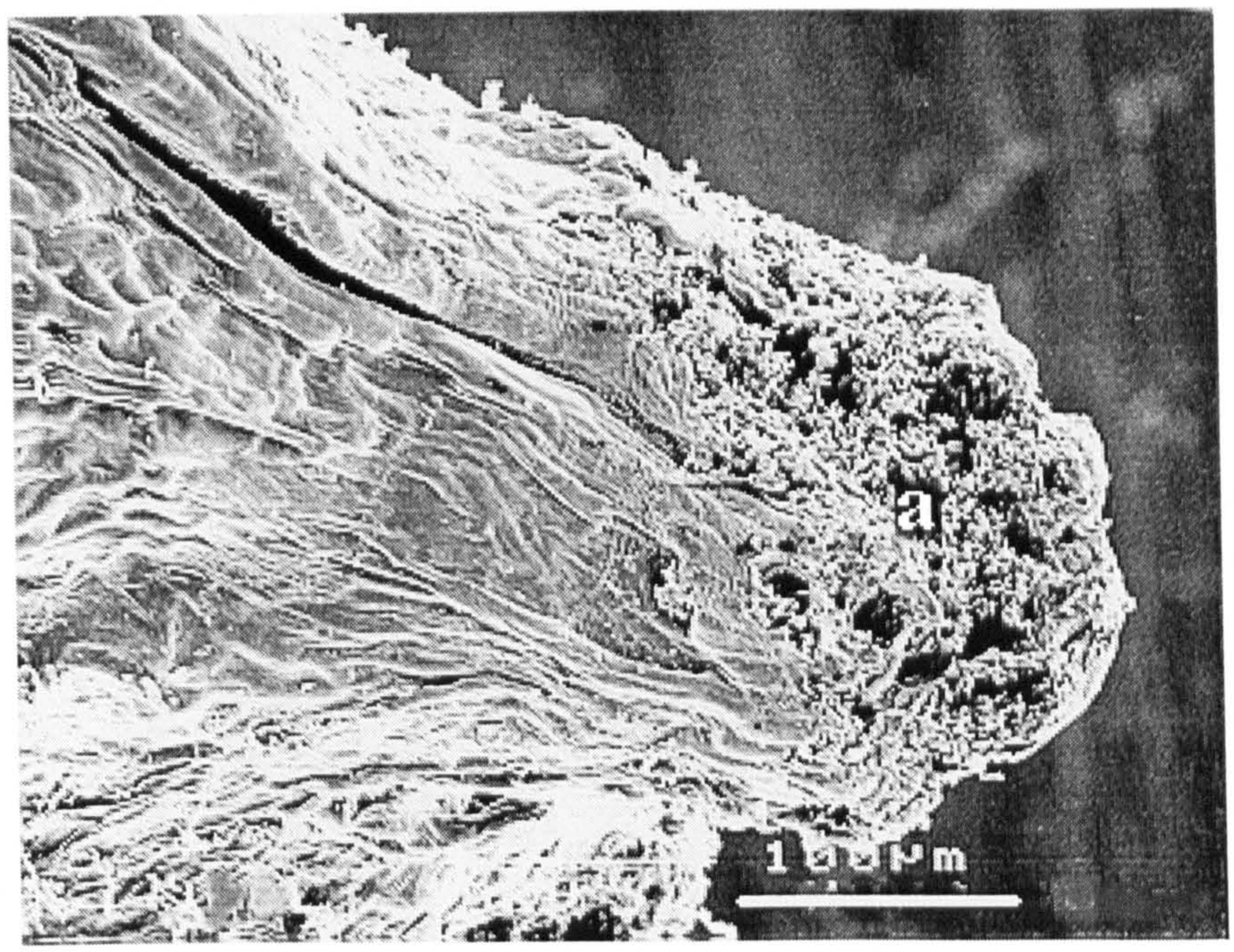


Plate 3.32 The tip from the seed in plate 3.31, magnified.

At higher magnification there is evidence of large numbers of microbial colonies (a).

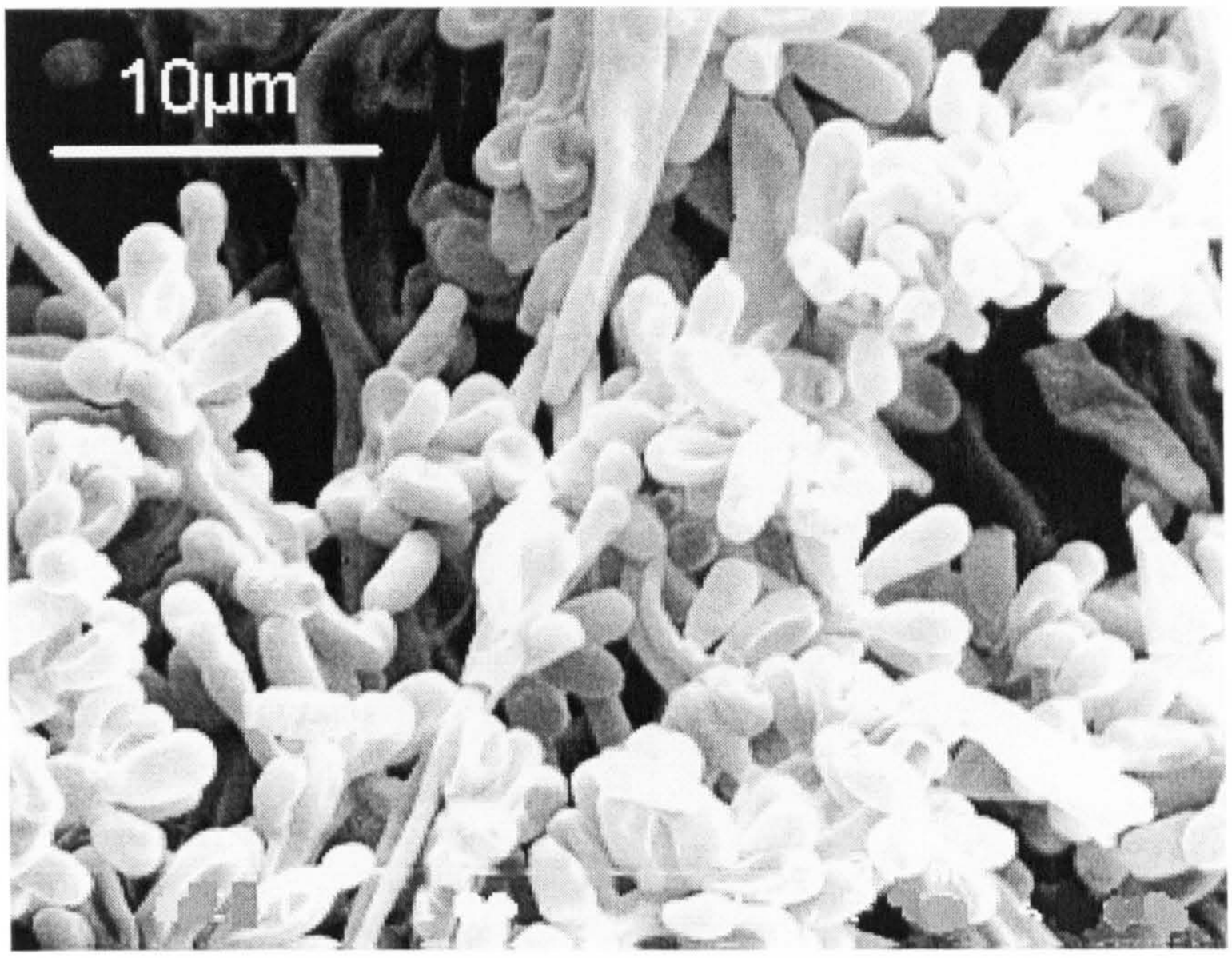


Plate 3.33 The tip of the *Rosa corymbifera* 'Laxa' seed in plate 3.32 magnified. This area was found to be covered in fungi. This could have been damaged during extraction from the hip thus rendering it more vunerable to microbial attack.

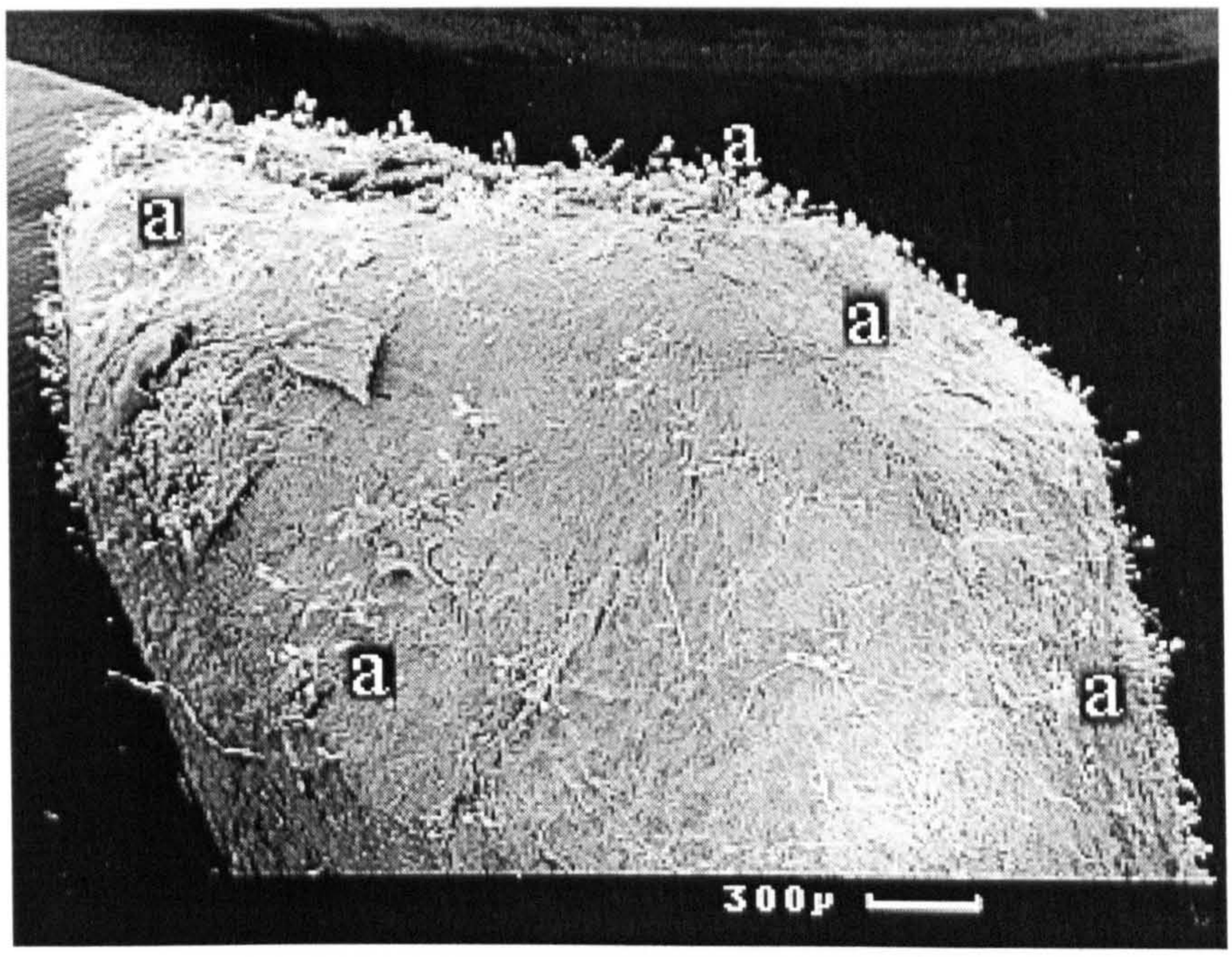


Plate 3.34 An air dried, untreated *Rosa corymbifera* 'Laxa' seed which was subjected to a sterile, humid environment for 48 hours.

Fungal colonies have formed across the entire outer surface of the seed coat (a). These colonies could only have originated from fungi already present on the seed when it had been air dried.

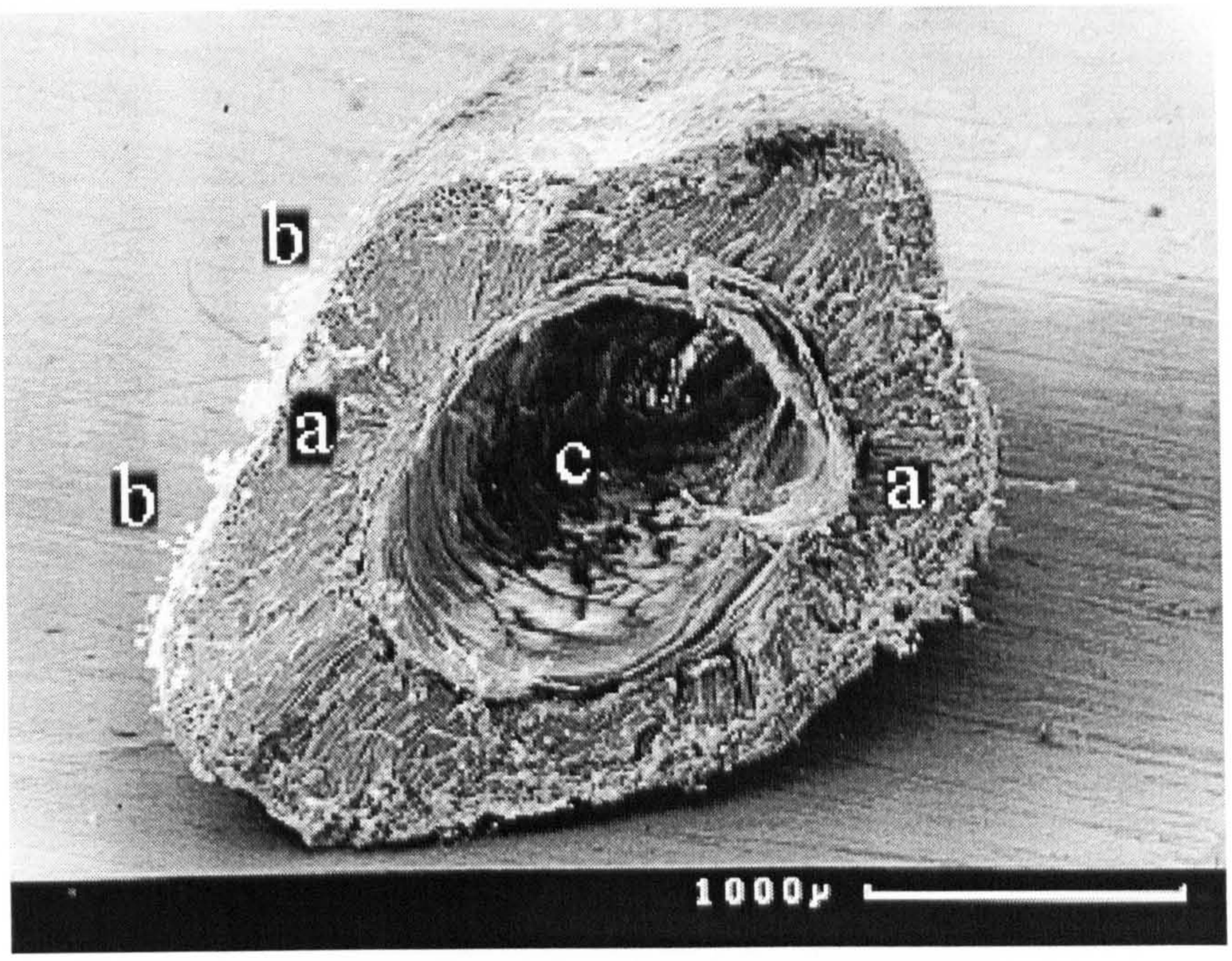


Plate 3.35 A cross section of a *Rosa corymbifera* 'Laxa' seed treated in the same way as that in plate 3.34.

A similar effect was found as with the whole seed. Colonies have also encroached across the cut face of the seed (a) as well as across the outer seed coat (b). The hole in the centre of the seed was where the embryo would have been (c).

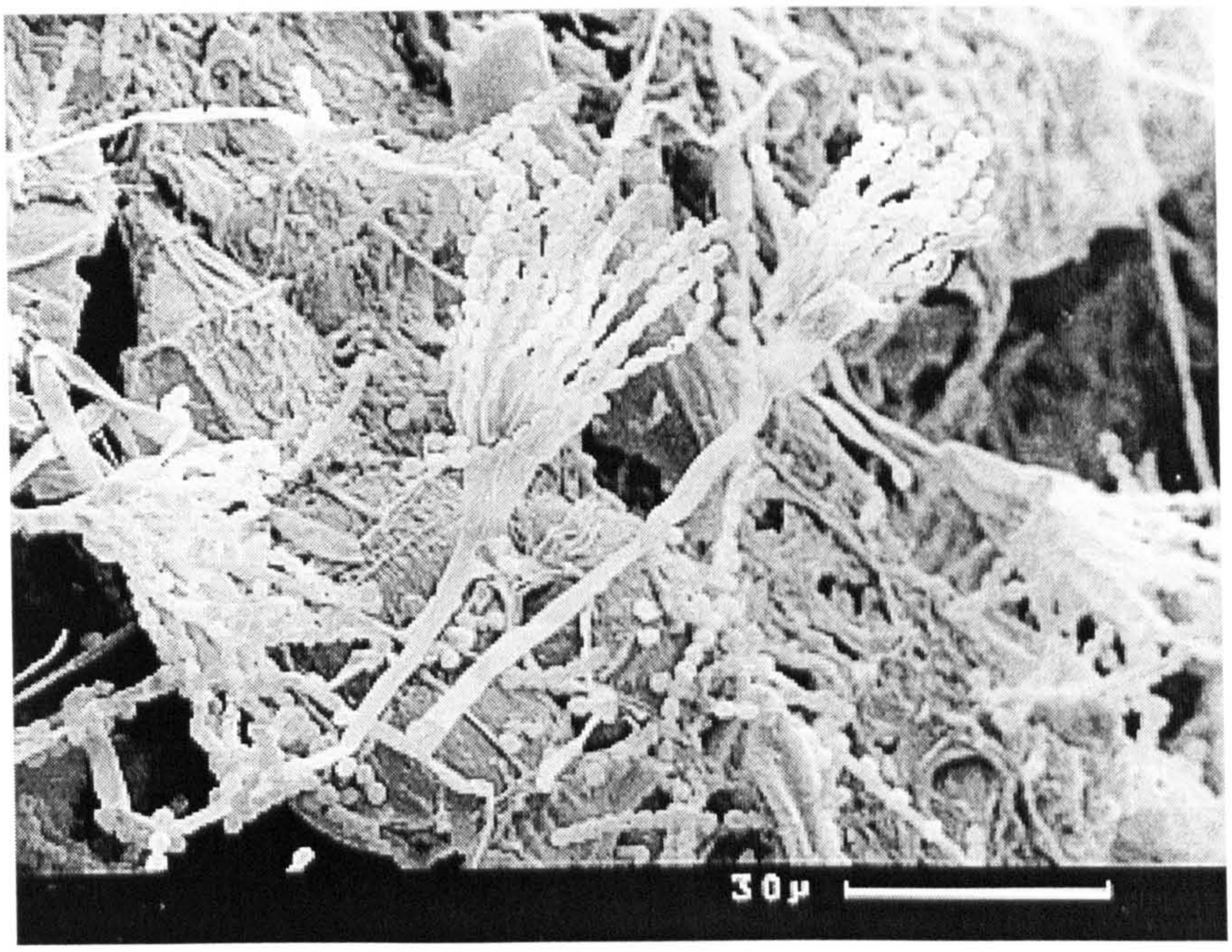


Plate 3.36 Magnified fungal colonies from plates 3.34 and 3.35.

Spores and fruiting bodies of a *Penicillium* species are clearly visible. These were present all over the seed surface after only 48 hours in a humid environment.

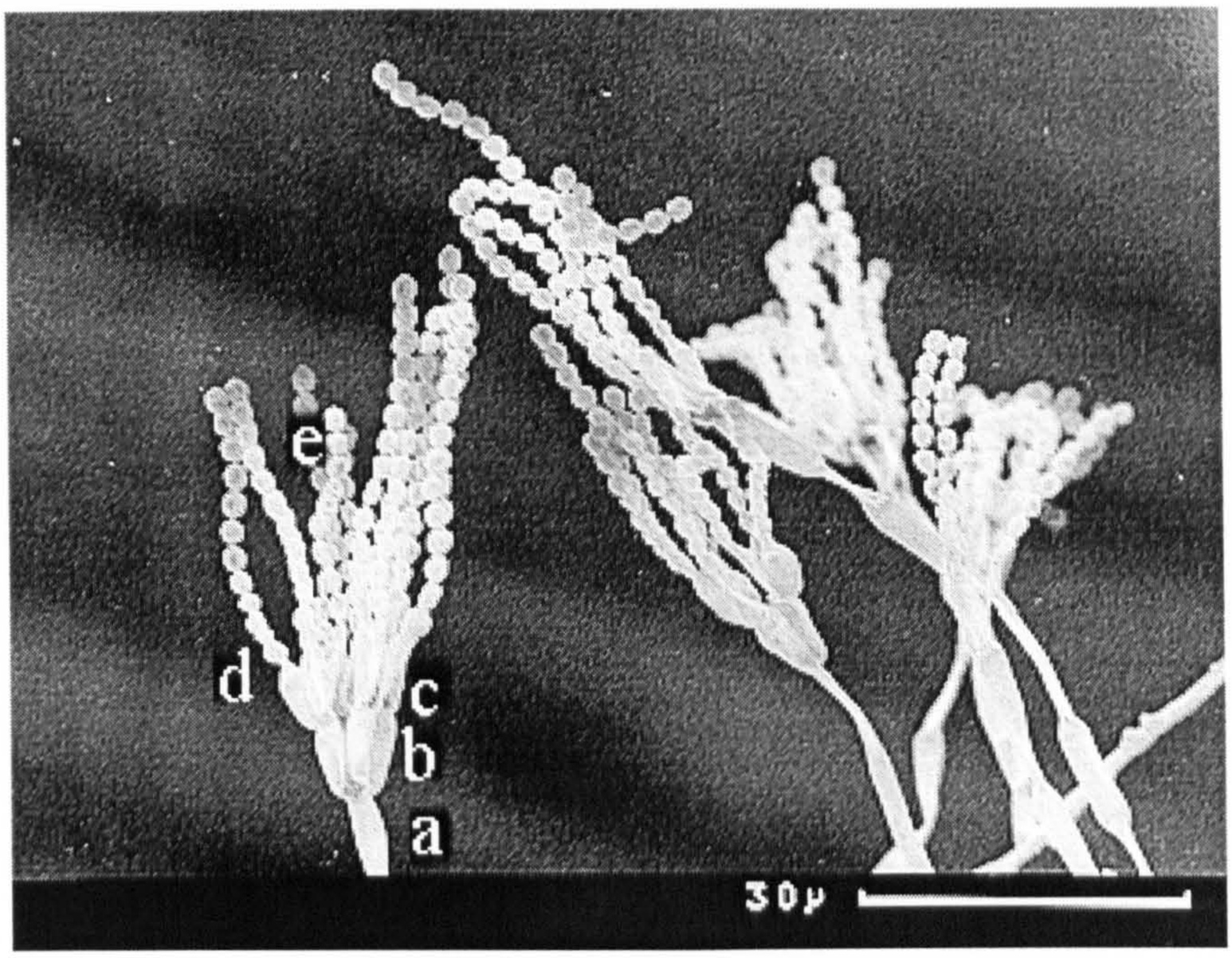


Plate 3.37 The individual fruiting bodies and spores on the seeds of *Rosa corymbifera* 'Laxa' in plates 3.34 and 3.35.

The detailed structures of the conidiophore (a), ramus (b), metulae (c), phialide (d) and spores (e) are clearly visible.

3.3.3 Discussion

Microorganisms were detected on untreated seed (plates 3.32 and 3.33) using Scanning electron microscopy. This would be expected as the seed has already been identified as carrying the microbial loading into the pretreatment mix (section 2.2). Initial levels of microorganisms were low, although it is worth remembering that it was air dried seed which had previously been washed during cleaning.

When samples of air dried seeds were placed into an environment conducive to microbial growth, the quantity detected increased both in terms of numbers and distribution. Fungal growth was found to cover the entire seed coat after only 48 hours (Plates 3.34 - 3.37). The incubation was carried out in sterile eppendorfs, thus only microbes present on the seed could grow and multiply. This protected, humid and warm environment would be similar to that found during the commercial and Garotta pretreatments. It is therefore not surprising that microbes flourish in such conditions.

3.4 Summary discussion

The studies carried out into the structure of *Rosa corymbifera* 'Laxa' seeds resulted in a detailed understanding of the structure of the seed coat. It has a complex, three layered pericarp, which encases the testa. Each layer is composed of cells which are orientated perpendicular to each other, producing a very hard, protective layer for the embryo.

At the end of the commercial pretreatment little impact had been made on *Rosa corymbifera* 'Laxa' in terms of breaking the hard seed coat. The seed coat remained intact with no physical changes being detected. However, pretreatment with Garotta resulted in an obvious colour change to the seed coat, likely to be caused by the fungi present in the pretreatment. The majority of seeds had also split open by the end of the warm period of incubation. These features could be used to determine whether a seed had been successfully pretreated i.e. whether it would germinate. They were therefore used as an aid to predict when seed had been treated for a sufficient length of time.

Scanning electron microscopy showed the presence of microorganisms on untreated seed. The numbers were relatively low, however, this would be sufficient to constitute the microbial loading described in section 2.2.2. Microbial growth quickly increased in a humid environment. These conditions which suit microbial growth were also experienced in both the commercial and Garotta pretreatments.

4. MICROBIAL CHANGES DURING PRETREATMENTS

Microbes have been found to be associated with *Rosa corymbifera* 'Laxa' seed (chapters 2 and 3). They are carried on the seed after extraction from the hips, and enter the pretreatment mix (section 2.2.2). During commercial pretreatment the seed remains physically unchanged (3.1.2) and germination is low (2.3.2). However, when Garotta is added, seed darkens in colour and splits (3.1.2). The majority will then germinate (2.3.2).

The microorganisms which have been shown to be present in the studies so far could be contributing to these two main effects - physical change and germination. Chapter 4 follows the development and progression of the microbial population during the different pretreatments of *Rosa corymbifera* 'Laxa'.

Four different approaches were adopted to study microbial changes during the pretreatment of *Rosa corymbifera* 'Laxa'. Each of the approaches listed below has individual merits; the results from each method can then be collated to give a full account of the overall microbial changes occuring during pretreatment.

- **Microbial counts** - the tried and tested serial dilution and pour plate technique to determine microbial numbers (Schlegel, 1993). This is a relatively simple technique which provides microbial counts as colony forming units (cfu's) as many colonies can result from one initial colony in the treatment. The method used was an adaptation of the dilution plate procedure described by Garrett (1981) as the soil dilution plate. The method has the advantages of being straightforward and allows many samples to be processed within a practical period of time.

  Its main disadvantage is one common to all such plating techniques; that is the selectiveness of the media used. No agar is optimum for all microorganisms, thus whilst one medium will favour fungi, it will not favour bacteria. To help overcome this two different media were used throughout these experiments, Potato Dextrose Agar (PDA) to select for fungi, and Nutrient Agar (NA) to select for bacteria.

- **Microbial activity** - using the fluorescein diacetate (FDA) assay to study metabolic capacity of microbes. This technique indirectly measures microbial activity by measuring the enzymes the microbes produce. The hydrolysis of FDA has the advantage of being simple, rapid and sensitive, and is particularly useful for comparative studies such as these (Schnürer & Rosswall, 1982). Many other methods are available to assess microbial activity, however they often have at least one major disadvantage in either being expensive, technically very sophisticated, time consuming, having a selective microbial range, an inability to detect living but inactive cells or requiring highly skilled personnel (see Swisher and Carroll (1980) for a summary review). Swisher and Carroll (1980) concluded that the FDA assay overcomes such shortfalls and the author found that many samples could be processed in a short space of time - especially important for the comparative nature of this thesis.

- **Microbial population** - assessing the degradative capacity of the microbes present. The cotton strip assay has been developed from its original use by the textile industry to being used as a biological assay by ecologists. It assess the cellulolytic microbial activity in soils (Sagar, 1988a). Thus it was an obvious assay to use in the study of microbial activity in the pretreatment of *Rosa corymbifera* 'Laxa' due to the suitability of the pretreatment medium and as the seed coat has a high cellulose content (section 3.1.2) which constitutes the main carbon source for the microbes. The assay is also relatively simple, not requiring expensive equipment, and many cotton strips can be processed in a day. They also have the convience of being able to be stored (frozen) if a delay in processing is required such as transport or time constraints.

- **Visual assessment** - studying seeds using scanning electron microscopy at different stages in the pretreatment. This was conducted to try to establish whether the microbes might be concentrated on particular parts of the seed and to visually distinguish potential species or groups of species present. It may also show whether different treatments have higher levels of microbes associated with the seeds.

Aim - to assess microbial numbers, activity and populations during the commercial and Garotta pretreatments of *Rosa corymbifera* 'Laxa', and to investigate the physical presence of microbes.

Objectives - to estimate the numbers of bacteria and fungi present during the warm period of the pretreatments.

To monitor microbial activity to give an indication of metabolic capacity of microorganisms in the pretreatment.

To assess the microbial population producing cellulase, a key enzyme in degradation of plant material.

To show the physical presence of microbes on seeds of *Rosa corymbifera* 'Laxa' during the pretreatments using scanning electron microscopy.

To show that microbes are present on the seeds during the commercial and Garotta pretreatments using scanning electron microscopy.

To use commercially available enzymes to try to degrade the seed coat.

4.1 Microbial numbers during pretreatments

Objective - to estimate the numbers of fungi and bacteria present during the warm period of the pretreatments.

4.1.1 Materials and Methods

One gramme samples were taken aseptically from the pretreatment mixes and placed into 9mls sterile tap water and shaken. Serial dilutions were made from the initial 1g samples. Dilutions were made down to 10^{-8} when appropriate (dilutions were made based on the previous time point results). Pour plates were made with 1.0ml of the particular dilution and either Nutrient Agar (NA) or Potato Dextrose Agar (PDA), to select for bacteria and fungi respectively. Once set, the plates were incubated at 25°C for 48 hours before colonies were counted.

In later experiments spread plates were used whereby 0.1ml dilutent was spread aseptically across the surface of pre-prepared agar plates. This method allowed for easier handling and preparation for large numbers of samples. This type of plate also provides a totally aerobic environment for the microbes to grow in. The pour plate method restricts oxygen as the microbes are 'encased' in agar - however only the size of colony is likely to be affected by this, not the actual growth. Therefore the microbial numbers obtained from both methods are comparable.

Three replicates for each treatment were set up. Three 1g samples were taken from each replicate and serial dilutions made based on estimations of likely microbial numbers. Plating was performed on the dilutions which would result in between 30 and 300 colonies growing on them. Three dilutions were plated out for each treatment, based on the microbial numbers recorded from the previous sampling time. Plates were made in triplicate for each of the two agars, NA and PDA. Thus for each treatment 18 agar plates were inoculated and assessed from samples taken from the pretreatments.

4.1.1.1 Microbial numbers during the first 14 weeks of pretreatment

During the course of this experiment, pretreatment *of Rosa corymbifera* 'Laxa' was carried out with three different treatments. The commercial pretreatment and Garotta pretreatment (1.0g added per 10g seed) were exactly as described in section 2.1.1. The third treatment had 0.5g Garotta per 10g moist seed added to it at the initial setup.

Incubation of the treatments lasted 24 weeks in all cases. The first 12 weeks were maintained at 25°C and the remaining 12 weeks at 4°C (as described in the methodology, section 2.1.1). Microbial numbers were assessed for the first 10 weeks for the experiment conducted with the three pretreatments (commercial, 1.0g Garotta and 0.5g Garotta).

The second experiment conducted had microbes counted for the first 14 weeks of the incubation, i.e. to include a time point during the cold period. Plates made from washings taken from samples taken from the cold (week 14) were incubated under normal conditions (25°C) and a second set were incubated at 4°C (labelled as 14a in the results) (the same incubation temperature as the cold period of pretreatment). This second set of plates was then incubated at 25°C after the initial incubation at 4°C (labelled as 14b in the results)

A higher rate of Garotta than 1.0g was not used as experiments (described in section 5.1) have shown no enhancement in germination rate over the 1.0g Garotta rate.

4.1.1.2 Fungal numbers during the warm period of pretreatment

A second pretreatment of *Rosa corymbifera* 'Laxa' was conducted to study fungal counts in the view of the results detailed in section 4.1.2.1. Fungal counts were made during the warm period of incubation, in particular to study the initial 3 weeks when previous studies had recorded the highest levels of fungi. Samples were taken every 2 days for the first 2 weeks, during which time peak numbers were recorded. Sampling was then performed after 21, 28, 35, 42, 56 and 70 days. The methodology was the same as that previously described (section 4.1.1.1). The commercial pretreatment and 1.0g Garotta pretreatments were studied.

4.1.2 Results

4.1.2.1 Microbial numbers during the first 14 weeks of pretreatment

Bacterial and fungal numbers increased with time in all pretreatments to reach a peak after 3 weeks (figures 4.1 and 4.2 respectively; corresponding statistical significance between the two pretreatments is shown in tables 4.1 and 4.2). These microbial numbers were greatly enhanced in the presence of Garotta compared to the commercial treatment. The enhancement of microbial numbers by Garotta was always greater when 1.0g Garotta was added to 10g moist seed than when only 0.5g was added.

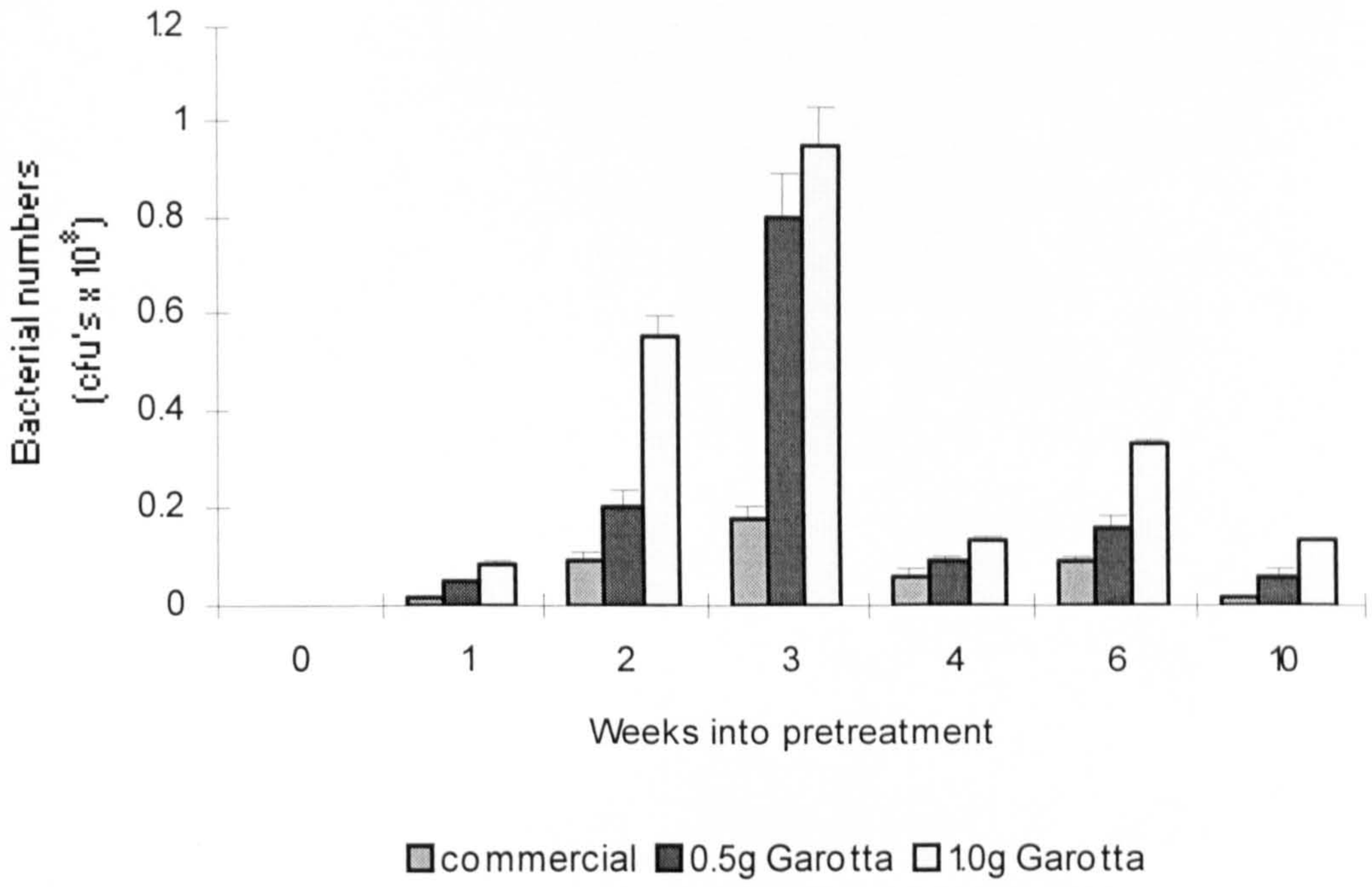


Figure 4.1 Bacterial counts during the first 10 weeks of the pretreatment of *Rosa corymbifera* 'Laxa'. Each treatment represents the average of 3 replicates; plates were triplicated per replicate. Standard error bars are shown for each point (where absent, the SE is too small to register).

Table 4.1 The level of significance between the commercial and 1.0g Garotta pretreatments for the bacterial counts (results shown graphically in figure 4.1). The raw data and statistical tables can be found in appendix 4.1.

	Week						
	0	1	2	3	4	6	10
Bacterial counts	nc	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%

nc = not calculated as no microbes were detected at initial setup.
ns = no significant difference.

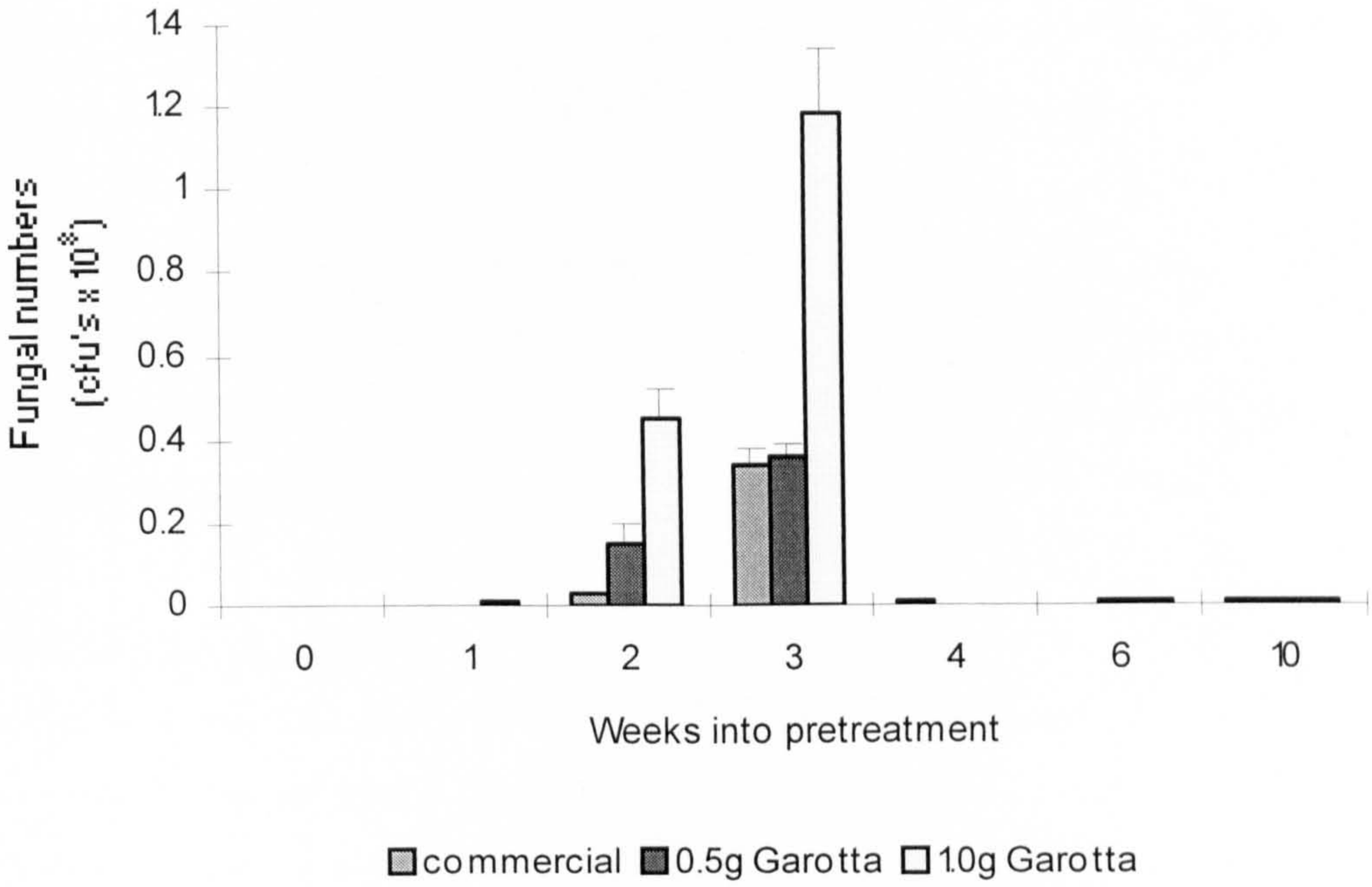


Figure 4.2 Fungal counts during the first 10 weeks of the pretreatment of *Rosa corymbifera* 'Laxa'. Each treatment represents the average of 3 replicates; plates were triplicated per replicate. Standard error bars are shown for each point (where absent, the SE is too small to register).

Table 4.2 The level of significance between the commercial and 1.0g Garotta pretreatments for the fungal counts (results shown graphically in figure 4.2). The raw data and statistical tables can be found in appendix 4.1.

	Week						
	0	1	2	3	4	6	10
Fungal counts	nc	0.1%	0.1%	0.1%	ns	0.1%	ns

nc = not calculated as no microbes were detected at initial setup.
ns = no significant difference.

Microbial counts are expressed as colony forming units (cfu's). This indicates that whilst discrete colonies may grow on the agar plates, one colony does not necessarily originate from one individual from the pretreatment. Many colonies could be produced from one individual fungus due to fragmentation during washings and plating, however comparisons are valid as all the samples are treated in the same manner.

The trend shown in the two graphs was one of rapid increase in microbial numbers up until week three of incubation for all treatments, followed by an equally rapid decline. This decline was much more appreciable with the fungal counts than the bacterial counts.

During the course of this experiment the microbial numbers were always higher for the Garotta pretreated seed than the commercial treatment. The 1.0g Garotta pretreatment also showed increased numbers over those of the 0.5g Garotta treatment.

One way analysis of variance (ANOVA) was carried out to determine the validity of combining replicates within pretreatments. No statistical differences were found within treatments except for week 10 in the 0.5g Garotta treatment and weeks 6 and 10 in the commercial treatment (see appendix 4.1).

Once this was established a one way analysis of variance was carried out between the commercial pretreatment and 1.0g Garotta pretreatment for both bacterial and fungal numbers. Samples taken at the setup of this experiment (time 0) produced no microbial numbers - probably as numbers at this point are very low (section 2.2.2). Therefore statistical analysis was not appropriate.

Tables 4.1 and 4.2 show the results of the statistical analysis which compares microbial numbers between the commercial pretreatment and 1.0g Garotta pretreatment. At each time point (week 1 - 10) the bacterial numbers were very highly significantly different between the pretreatments. Fungal numbers were also very highly significant between the pretreatments for weeks 1 - 10, with the exception of weeks 4 and 6 where no significant difference was found.

The results of the microbial number counts for the experiment which also sampled the pretreatments in the cold period are shown in figures 4.3 and 4.4. The numbers are in the same order of magnitude as those found previously (figures 4.1 and 4.2). Sampling occurred after 3, 6, 9, 12 and 14 weeks. The 1g samples taken at 3, 6, 9 and 12 weeks were washed as usual and spread plates made. The plates were then incubated at 25°C for 48 hours. However, the samples taken at 14 weeks were treated slightly differently. Twice as many plates were made as normal from the serial dilutions, half were incubated at 25°C and the others were incubated at 4°C - the temperature at which the pretreatment mix was maintained at throughout the cold period. Counts were made from both sets of plates. No colonies were found after this incubation in the cold, so these plates were transferred to 25°C for 48 hours. Counts were made and found to be the same as the samples which were first incubated at 25°C.

Both fungal and bacterial numbers followed the same trend, i.e. decreasing as the pretreatment continued. The first sample time was after 3 weeks and previous experiments showed that this was the when maximum microbial numbers were found (figures 4.1 and 4.2). The graphs therefore show the decline in numbers following this, to a low after 12 weeks. On transfer to the cold, numbers continued to decline.

However, microbial growth stopped in the cold even though microbes remain viable at this temperature. This is shown by the results obtained by incubating the same sample dilutions under 25°C (labelled as 14) and 4°C (labelled as 14a) conditions. No microbes grew on plates incubated at the lower temperature, but growth did occur at the higher temperature. Once the plates with no microbial colonies (initially incubated at 4°C) were transferred into 25°C (labelled as 14b), growth resumed. Therefore, whilst microbes cease to grow during the cold period of pretreatment, they do remain viable.

The data were statistically analysed using a one way analysis of variance. There was significant difference between all Garotta and commercial pretreatments for both bacterial and fungal numbers. These findings are tabulated below the relevant graph (tables 4.3 and 4.4).

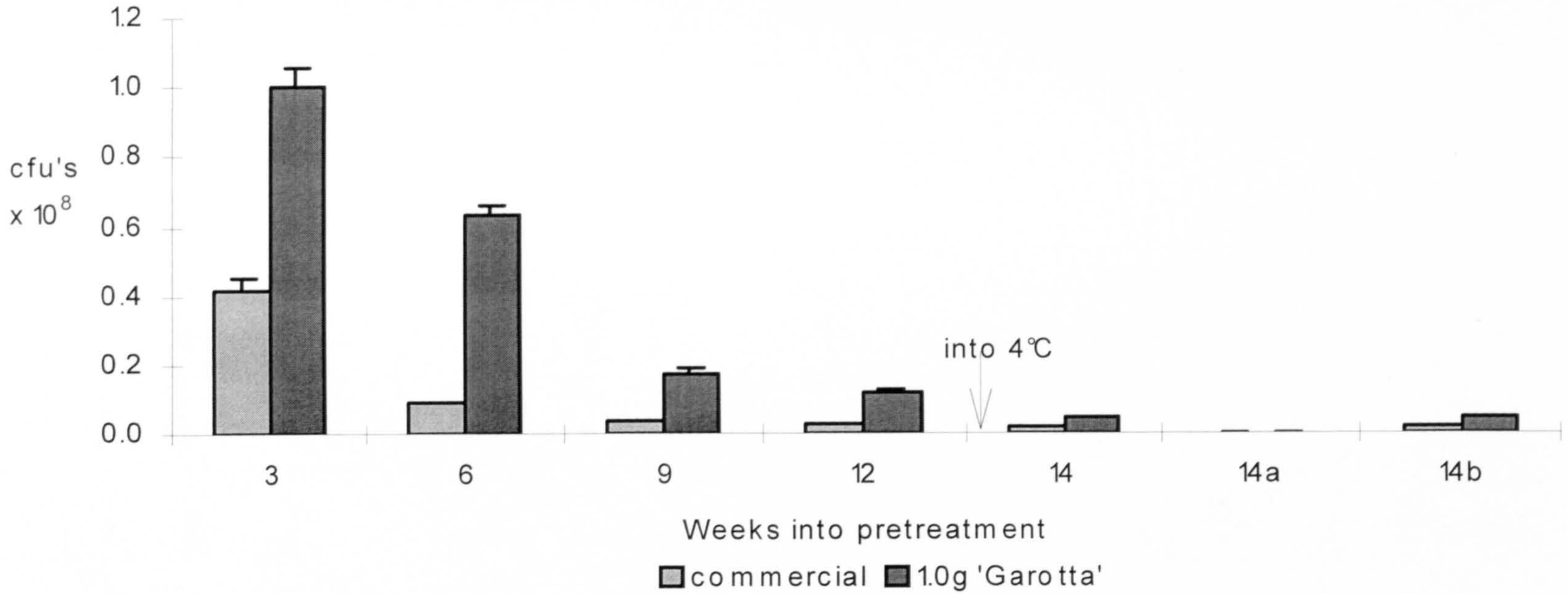


Figure 4.3 Bacterial counts during the first 14 weeks of the pretreatment of *Rosa corymbifera* 'Laxa'.
Each treatment represents the average of 3 replicates; plates were triplicated per replicate.
Standard error bars are shown for each point (where absent, the SE is too small to register).

Table 4.3 The level of significance between the commercial and 1.0g Garotta treatments for the 14 week bacterial counts (shown graphically in figure 4.3). The raw data and statistical tables can be found in appendix 4.2.

	Week						
	3	6	9	12	14	14a	14b
Bacterial counts	0.1%	0.1%	0.1%	0.1%	0.1%	na	0.1%

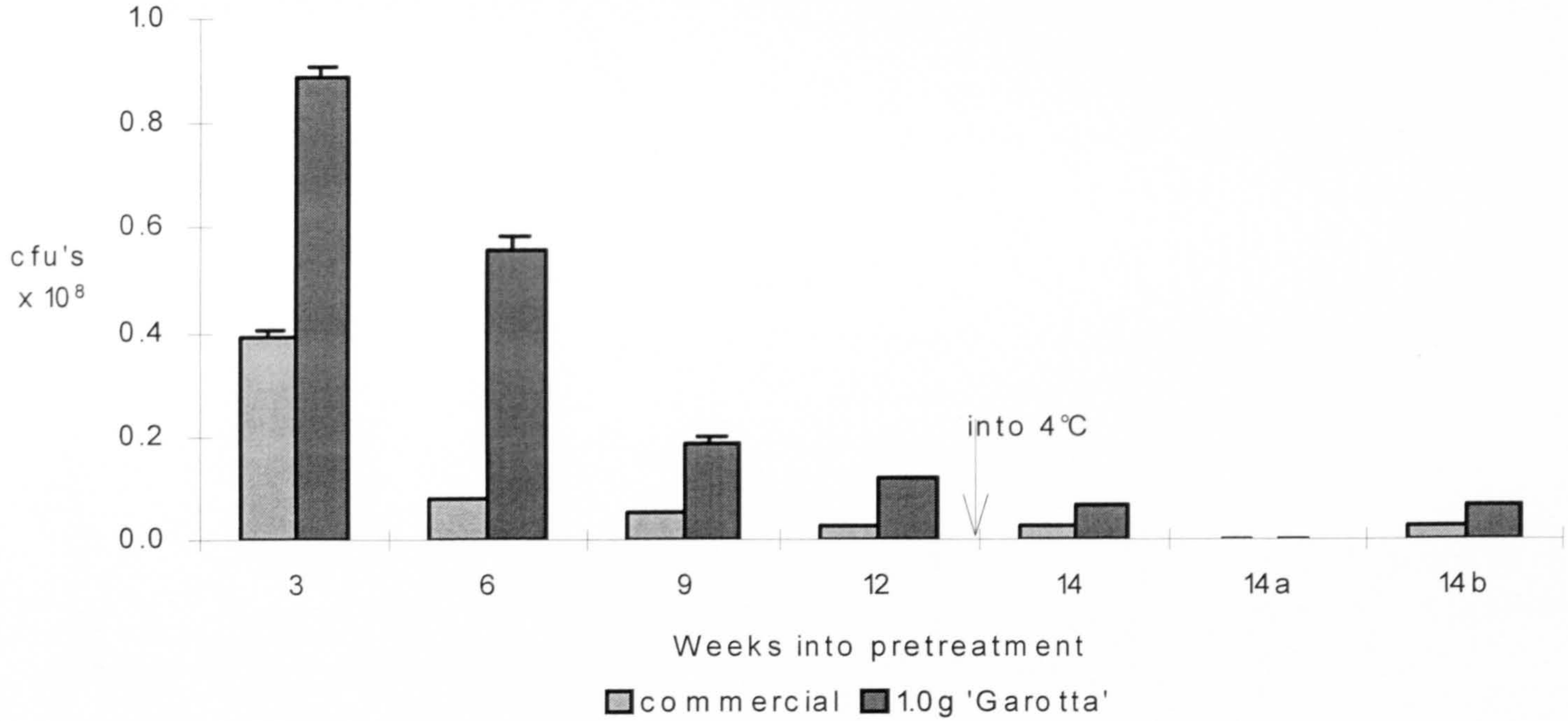


Figure 4.4 Fungal counts during the first 14 weeks of the pretreatment of *Rosa corymbifera* 'Laxa'.
Each treatment represents the average of 3 replicates; plates were triplicated per replicate.
Standard error bars are shown for each point (where absent, the SE is too small to register).

Table 4.4 The level of significance between the commercial and 1.0g Garotta treatments for the 14 week fungal counts (shown graphically in figure 4.4). The raw data and statistical tables can be found in appendix 4.2.

	Week						
	3	6	9	12	14	14a	14b
Fungal counts	0.1%	0.1%	0.1%	0.1%	0.1%	na	0.1%

4.1.2.2 Fungal numbers during the warm period of pretreatment

The results of the fungal counts in this experiment are shown graphically in figure 4.5. A similar trend to the fungal numbers was found to that described for the previous experiments (figures 4.1 - 4.4), although the peak activity was found between day 8 and 14. Whilst this was earlier than the previous experiments, fungal numbers were in the same order of magnitude for both sets of results.

Fungal numbers were always higher in the 1.0g Garotta pretreatment than the commercial pretreatment, with the exception of day 35 and day 56. Significant differences (one way ANOVA) were found between the pretreatments for days 4, 8, 10, 12, 14, 21 and 70 (table 4.5). These correspond to the time points on the graph (figure 4.5) where there is a large difference between the fungal numbers of the commercial pretreatment and the 1.0g Garotta pretreatment.

Some significant differences were found within pretreatments, however only in two cases (days 4 and 21) did the within pretreatment significance exceed the between pretreatment significance. Where no significance was found between pretreatments it was sometimes found that there was significance within pretreatments. Thus, where the fungal numbers are different between the commercial pretreatment and the 1.0g Garotta pretreatments, statistically significant differences are not found due to the variation experienced within those pretreatments.

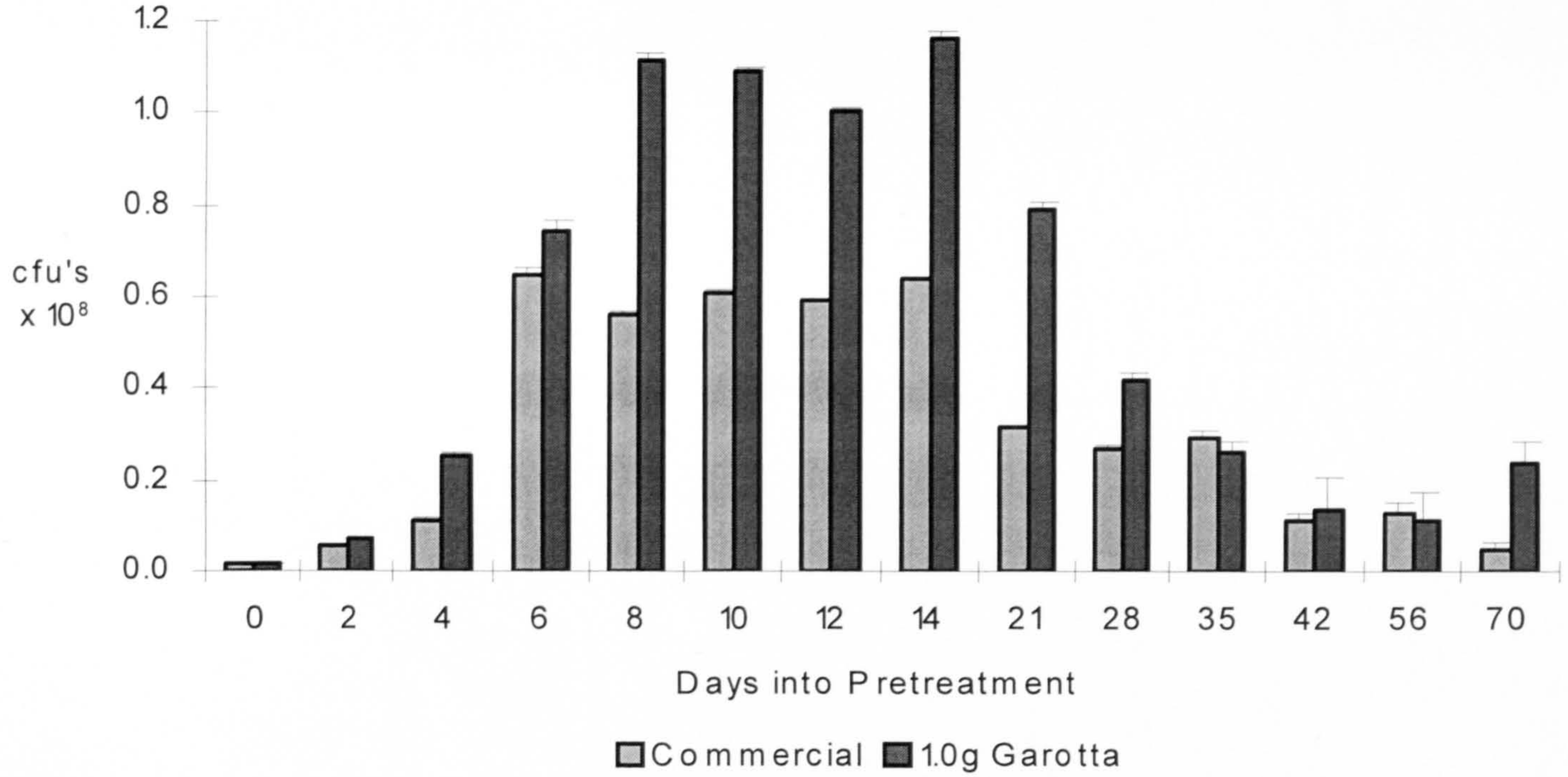


Figure 4.5 Fungal counts during the first 70 days (10 weeks) of the pretreatment of *Rosa corymbifera* 'Laxa'.
Each treatment represents the average of 3 replicates; plates were triplicated per replicate.
Standard error bars are shown for each point (where absent, the SE is too small to register).

Table 4.5 The level of significance between the commercial and 1.0g Garotta pretreatments for the 70 day fungal counts (shown graphically in figure 4.5). The raw data and statistical tables can be found in appendix 4.2.

	Day													
	0	2	4	6	8	10	12	14	21	28	35	42	56	70
Fungal counts	ns	ns	5%	ns	5%	1%	5%	1%	1%	ns	ns	ns	ns	1%

ns = no significant difference

4.1.3 Discussion

The graphs in sections 4.1.2.1 and 4.1.2.2 correspond to a surge in microbial numbers from the initial setup (time 0) to a peak after two to four weeks. As temperature and moisture were not limiting, it is likely that the little microbial build up seen in the commercial treatment was due to the lack of a nutrient source.

Microbial growth is dependent upon the nutrient status of its immediate environment (Schlegel, 1993). When nutrient starved, microbial growth becomes limited. Especially important is the C/N ratio. Where this is high the microbial growth is severly impaired (Allsopp & Seal, 1986). In the commercial treatment the C/N ratio is potentially very high. Woody tissue (tissue containing sclerenchyma) has a very high C/N ratio (Allsopp & Seal, 1986) and therefore little or no available nitrogen is present in the seed. As no nitrogen source is added to the commercial pretreatment medium then microbial growth will potentially be severely limited by the lack of nitrogen.

However, a nutrient source was provided in the other pretreatments by the addition of Garotta. This was previously found to be high in nitrogen and other nutrients (see section 2.3 for analysis). The 1.0g rate of Garotta pretreatment produced increased microbial numbers over the half rate (0.5g), probably due to the larger quantity of nutrient source added at the setup of the pretreatments. It was therefore likely that the amount of Garotta added was a limiting factor for microbial growth within this system (numbers fall off after 3 - 4 weeks). However it is not a limiting factor for seed germination (section 2.3 for results and discussion). The nitrogen component of Garotta is in the form of water soluble ammonium salts, and consitutes 22% of the Garotta (section 2.3.2). Thus in a 'typical' Garotta pretreatment consisting of 100g seed, 250g vermiculite and 10g Garotta, ammonium salts represent 0.6% of the total. The standard defined culture medium for growing microorganisms contains 0.1% ammonium salts as the nitrogen component (Madigan *et al*, 1997). It would therefore appear that the Garotta pretreatment has more than an adequate nitrogen content for microbial growth. Obviously the amount contained in the culture medium is only enough for a limited growth period, so although there is much more in the Garotta pretreatment at the outset, it will be depleted and consequently microbial growth will become limited (after weeks 3 - 4).

A potential stress which could have occurred when conducting these experiments was oxygen deprivation during incubation of the agar plates. Pour plates enclose the microbes in agar and this could cause oxygen stress to the microbes. However there is little evidence that this would occur, as thin layers of agar are amply supplied with oxygen (Schlegel, 1993). Oxygen deficiency or stress is only likely to occur in situations where surface area : volume ratios are low e.g. liquid culture or over prolonged periods of time where aeration is absent (Schlegel, 1993). The pour plates prepared for these experiments were relatively thin and only incubated over 48 hours. Furthermore, even if stressed, growth is still likely to proceed, and as only numbers of microbes were collected, symptoms of oxygen stress which would be noticeable later in the life cycle are not relevant. Numbers of microbes obtained with pour plates (4.1.2.1) were very similar to those obtained when spread plates (microbes on surface of plate) were used in later experiments (4.1.2.2), offering further evidence that oxygen stress had little if no bearing on the results.

Statistical analysis on the data showed that there were statistical differences between the microbial numbers found in the commercial pretreatment and the 1.0g Garotta pretreatment. It can be concluded that the addition of Garotta to the pretreatment of *Rosa corymbifera* 'Laxa' enhanced the numbers of microbes compared to those found in the commercial pretreatment.

Although significant differences were not found between every set of data compared, this was not unexpected considering the nature of the data which was analysed. Samples were taken randomly from the 6 replicates of each treatment. There is likely to be variation found with this method as microbial growth will vary within the media in each replicate. Thus sampling one small area may not give a measurement of biomass throughout the entire medium. There is also likely to be variation between different replicates of the same treatment as biological systems do not always behave equally.

4.2 Microbial activity during pretreatment

The previous section looked at the microbial numbers during the commercial and Garotta pretreatments of *Rosa corymbifera* 'Laxa'. However, as mentioned in the results, these are based on colony forming units (cfu's). It is therefore not an exact measure of numbers and is likely to be an over estimation. This section looks at the activity of the microbes rather than numbers.

Objective - to monitor microbial activity to give an indication of metabolic capacity of the micro-organisms in the pretreatment mixes.

Many techniques have been developed to estimate overall microbial activity in soil, litter and water (Swisher & Carroll, 1980; Schnürer & Rosswall, 1982; McCarthy, 1987). However, they all suffer from inherent difficulties and/or considerable technical sophistication and expense (Swisher & Carroll, 1980). When using these techniques for microbial biomass estimation, the assumption is made that the chemical component and total cell biomass have a relatively constant ratio (Swisher & Carroll, 1980). Even if this assumption cannot be met, the values obtained can still be used as an index of biomass or relative activity.

The fluorescein diacetate (FDA) assay provides a 'simple, sensitive and rapid' technique to estimate microbial activity (Schnürer & Rosswall, 1982; McCarthy, 1987). Fluorescein diacetate (3', 6'-diacetylfluorescein (FDA)) is a non fluorescent substrate hydrolysed by a number of different enzymes such as proteases, lipases and esterases (Guilbault & Kramer, 1964; Rotman & Papermaster, 1966) present in all viable microbial cells (McCarthy, 1987). Fungal hyphae are only capable of FDA hydrolysis when viable - thus only metabolically active microbial cells will hydrolyse FDA (Söderström, 1977). This method has been applied to fungi (Söderström, 1977; Söderström & Erland, 1986), yeasts (Paton & Jones, 1975), bacteria (Lundgren, 1981; Chrzanowski *et al*, 1984) and total biomass (Swisher & Carroll, 1980).

FDA is hydrolysed by the enzymes to release fluorescein which can be quantified by fluorometry or spectrophotometry. This technique was first shown to be effective in measuring total microbial activity in soil samples by Schnürer & Roswall (1982) and on coniferous needle surfaces by Swisher & Carroll (1980).

Review of the literature found that FDA has not only been used to measure microbial activity but has also been used to test the viability of orchid seeds (Pritchard, 1985), pollen viability (Zuttini *et al*, 1996) and plant shoot apical meristem viability (Popov & Vysotskaya, 1996). The method used for this study therefore had two approaches - direct microbial activity on samples from the pretreatments and the use of cotton squares to assess microbial activity directly associated with the fabric (Smith & Maw, 1988; McCarthy, 1987). The latter method was designed to measure activity of the microbes whilst excluding any potential activity from the seed.

4.2.1 Materials and Methods

1g samples from the pretreatment mix of the commercial pretreatment and Garotta pretreatment (described in sections 2.1.1 and 2.3.1 respectively) were processed according to the following protocol. 1g of well mixed sample (referred to in the text as 'sample') was transferred to a 50ml conical flask and 10ml of sodium phosphate buffer (pH 7.6) was added to each. FDA hydrolysis was initiated by the addition of 200µl of a 1 mg/ml FDA solution (to give a final FDA dilution of 20 µg/ml). The flask was capped with parafilm and incubated at 37°C for 30 min in a shaking water bath (approx. 180 strokes/min).

The sample was removed from the water bath and 10ml of acetone added to terminate the reaction. 1.5ml of fluid sample was centrifuged at 13000 rpm (micro centrifuge) for 30 seconds before being decanted into a glass cuvette. The absorbance of the sample supernatant held in the 10mm path length cuvette was measured at 490nm using a spectrophotometer. A blank was prepared using sodium phosphate buffer, acetone and FDA in the proportions previously described and absorbance measured in a matched glass cuvette.

After six weeks, small pieces of burial test fabric ($2cm^2$), (described in full in section 4.3), were also inserted into the pretreatment mix to allow FDA analysis. This method had been previously used effectively by Smith and Maw (1988). This was to give a measure of the enzymes within the pretreatment mix but excluding the seed which the 1g samples may contain.

The components which made up the pretreatments were also tested using the FDA assay; i.e. vermiculite, seed and Garotta, using equivalent masses found in a 'typical' 1g sample. The equivalent mass of each component was calculated from the proportions of vermiculite, seed and Garotta used when the 1.0g Garotta pretreatment of *Rosa corymbifera* 'Laxa' commenced. The mass sampled was the proportion of each component which would be found within a 1g sample. i.e. if 10g of seed were treated according to the method described in section 2.1.1 and 2.3.1, then 25g vermiculite, 10g moist seed and 1g Garotta would be mixed together. As a proportion each would represent 0.7g, 0.27g and 0.03g respectively (see results, 4.2.2 for complete table).

Also tested were samples of seed taken four weeks into each treatment (commercial and Garotta), i.e. when microbial numbers were at their maximum, and samples of seed which had been autoclaved (no longer viable). A positive control was used to give an indication of the activity of agar cultured fungal growth. For this, two day old cultures of *Rhizopus* spp. were used, and plugs of these were tested using the FDA assay. The 2 and 4 plug samples were triplicated and the average used as a comparison.

As an example to allow comparison with the estimation of biomass, figures are also quoted from a yeast calibration for the FDA assay (Williams, 1993). These figures give an absorbance value and the equivalent cell concentration of yeast cells (cells/ml) which equates to yeast biomass.

The values used in the results are plotted against µg fluorescein, having converted the absorbance reading taken at 490nm using the calibration curve in appendix 4.3.

4.2.2 Results

Table 4.6 shows the results of calculating the equivalent mass of each component (i.e. vermiculite, seed and Garotta) for a 'typical' 1g sample taken from a 'typical' pretreatment mix. The mass of the seed shown in this table (0.27g) was used for all the FDA assay tests of seed listed in table 4.7 (the autoclaved seed, commercially pretreated seed and Garotta pretreated seed).

Table 4.6 The equivalent mass of each component of the pretreatment mix found in a 'typical' 1g sample.

Component of pretreatment	equivalent mass of sample (g)
vermiculite	0.70
Garotta	0.03
seed	0.27

The figures listed in the table below (table 4.7) show the results of testing samples of the pretreatment components for fluorescein activity. FDA hydrolysis was not detected in the vermiculite or autoclaved seed (the reading of 0.001 quoted in the table below is the average of four readings, two of which were 0.000. The accuracy of the spectrophotometer is such that readings as low as 0.001 cannot be classed as a positive absorbance). Garotta, freshly harvested seed and commercially treated seed showed low levels of activity with Garotta treated seed having the highest level of activity. However, none of the activities can be considered particularly high when compared to the 4 plug positive control culture of *Rhizopus* or the yeast cell suspensions which were used.

Table 4.7 Absorbance readings following the FDA assay on samples from the pretreatment mix. Also included are the results for the *Rhizopus* cultures and yeast cell suspensions. The absorbance has been converted to µg fluorescein using the calibration curve (see appendix 4.3).

	Absorbance @ 490nm	µg fluorescein
vermiculite	0.000	0.0
Garotta	0.011	1.0
untreated seed	0.012	1.1
autoclaved seed	0.001	0.1
commercially treated seed	0.005	0.5
Garotta treated seed	0.110	11.6
Rhizopus; 2 plugs	0.074	7.8
Rhizopus; 4 plugs	0.383	40.6
Yeast* (2×10^7 cells)	0.09	9.5
Yeast* (1×10^8 cells)	0.54	57.2

* data taken from Williams, 1993

The results of using the FDA assay on samples and cotton squares taken during the commercial and Garotta pretreatments are shown in figures 4.6 and 4.7.

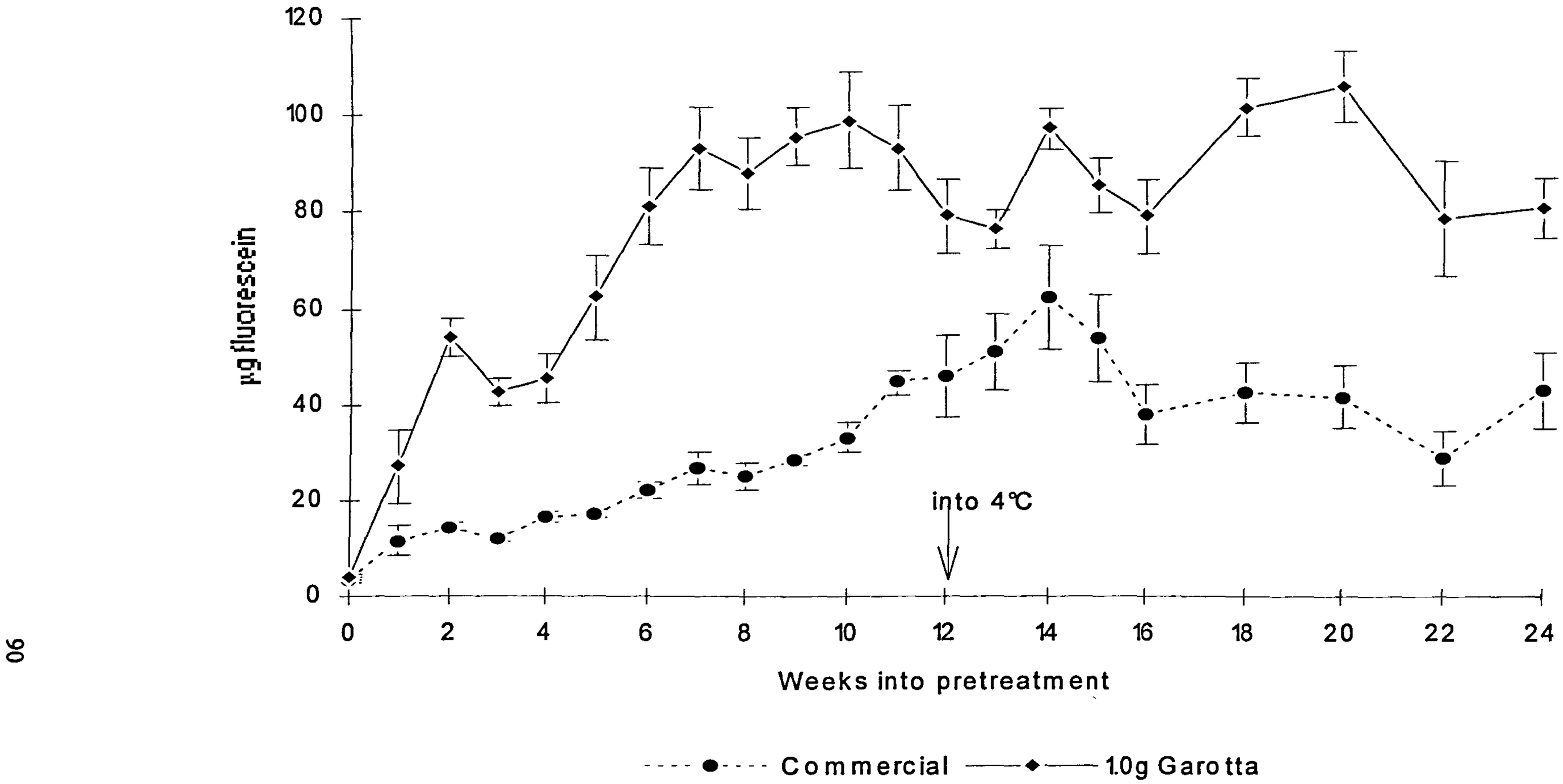


Figure 4.8 FDA results for the 1g samples taken during the 24 week commercial and Garotta pretreatment of *Rosa corymbifera* 'Laxa'.
Each data point represents the average of the means of two replicates (3 samples in each) from each treatment (see table 4.6 for statistics). Standard error bars are shown for each point (where absent, the SE is too small to register).

Table 4.8 The level of significance for the 1g samples between the commercial and 1.0g Garotta pretreatments for the FDA assay (shown graphically in figure 4.8). The raw data and statistical tables can be found in appendix 4.4.

	Week																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	18	20	22	24
1g samples	ns	ns	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	5%	5%	5%	5%	0.1%	0.1%	1%	1%	1%

The results clearly show that the Garotta treatment always showed a much higher microbial activity than the commercial treatment. Initially samples from both the commercial and Garotta pretreatments show little FDA activity, i.e. negligible microbial activity. However, within the first week both showed increased activity, the Garotta treatment to a greater extent. This trend continued throughout the warm period of incubation, reaching a peak at 10 weeks before the samples were transferred into the cold. During the cold period of incubation there was no marked decrease in microbial activity as measured by FDA activity.

Statistically significant differences were found between the commercial pretreatment and 1.0g Garotta pretreatment for all weeks except week 0 and week 1 (see table 4.8). Some significant difference was found within treatments, however the level of significance was always greater between the commercial pretreatment and 1.0g Garotta pretreatment except in week 14 for the commercial pretreatment and week 15 for both pretreatments.

The results in figure 4.9 are for the FDA assay which was performed on cotton squares which had been placed into the pretreatment commencing in week six. These were left in for 7 days then removed and tested for FDA activity. The activity measured for these samples would only measure that of microbial activity on the cotton, and not the seed.

The activity on the cotton squares was very low for the commercial pretreatment for the first 4 weeks but then increased slightly for the remainder of the warm period. The Garotta treatment showed a much higher FDA activity, and therefore microbial activity, throughout the course of the warm period than the commercial treatment.

Once the treatments were placed into the cold, activity fell back to a very low, if not negligible, level with both treatments. This effect was most noticeable in the Garotta treatment, where the FDA activity fell from 55 to 5 μg in one week.

The difference in activity between the commercial pretreatment and 1.0g Garotta pretreatment was statistically significant for all weeks the cotton squares were used (see table 4.9). There was some significant difference found within the pretreatments, week 10 for the 1.0g Garotta pretreatment and weeks 11-14 and 22 for the commercial pretreatment. However at all times the level of significance was higher between the pretreatments than within.

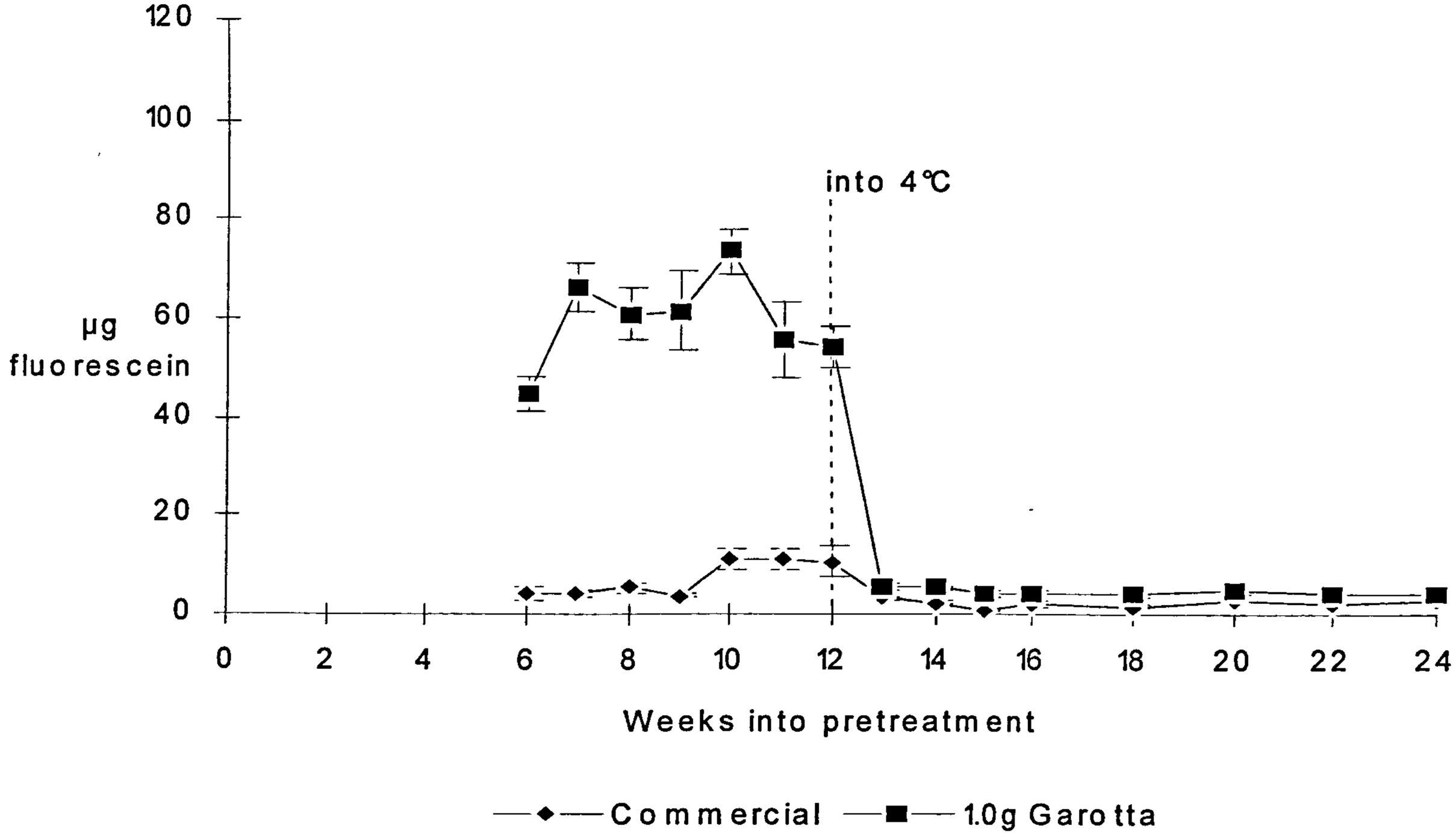


Figure 4.9 FDA results for the cotton squares taken during the last 18 weeks of the commercial and Garotta pretreatment of *Rosa corymbifera* 'Laxa'.
Each data point represents the average of the means of two replicates (3 samples in each) from each treatment. Standard error bars are shown for each point (where absent, the SE is too small to register).

Table 4.9 The level of significance for the cotton squares between the commercial and 1.0g Garotta pretreatments for the FDA assay (shown graphically in figure 4.9). The raw data and statistical tables can be found in appendix 4.4.

	Week															
	0 to 5	6	7	8	9	10	11	12	13	14	15	16	18	20	22	24
cotton squares	n/a	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	5%	0.1%	1%	0%	0.1%	1%

4.2.3 Discussion

The FDA assay has been used to determine active microbial biomass (Swisher & Carroll, 1980). Perhaps more relevant to this study is its use to determine total microbial activity (Schnürer & Roswall, 1982). These workers concluded that the determination of FDA hydrolysis would prove particularly useful for comparative studies in natural habitats. It is the comparative nature between the commercial and Garotta pretreatments where this technique has proved most useful in this thesis.

The results in figures 4.8 and 4.9 clearly show that FDA hydrolysis, and therefore microbial activity, was always greater with the Garotta pretreatment than the commercial pretreatment. The activity recorded originated from viable microbes within the pretreatment mix. These microbes produce the enzymes which cleave FDA to release fluorescein. Thus when a sample of the medium was taken, the resulting activity came from the enzymes which had been excreted from the microbes and from those enzymes present within the microbes.

The microbial activity was consistently higher when Garotta had been added to the pretreatment. Samples taken weekly from setup increased in activity to a maximum at 10 . weeks (94µg fluorescein) followed by a plateau effect i.e. little variation in activity from week to week. There was no noticable change in activity once the pretreatments were placed in the cold. The same trend was seen with the commercial pretreatment samples, although values were much lower (maximum reached in the warm of 42µg fluorescein was in week 11) than the Garotta pretreatment.

To attempt to quantify the FDA activity into microbial numbers, a comparison can be made with yeast cells. Table 4.7 gives values of fluorescein activity for viable yeast cell suspensions and *Rhizopus* spp. The $1x10^8$ yeast cell suspension gave a value of 57.2µg fluorescein whilst the 4 plugs of *Rhizopus* culture gave a value of 40.6µg. Therefore, for example, if the samples taken in this experiment contained only yeast then an approximate figure of $2x10^8$ viable cells could be extrapolated as the number of microbial cells present at the peak of FDA activity seen in figure 4.8. This is based on yeast grown in culture so . can only be used for comparative purposes.

The cotton square results (figure 4.9) followed a similar pattern to the 1g samples during the warm period of the pretreatment, although activity was always lower. Once the pretreatments were placed into the cold, activity measured on the cotton reduced dramatically to a minimal level (10 to 3µg for the commercial pretreatment and 52 to 5µg for the Garotta pretreatment). The explanation for this could be that whilst the microbes remain viable at lower temperatures (hence the continuance of high activity with the 1g samples), production of extracellular enzymes is suspended. Therefore little would be picked up by the cotton. As fresh cotton squares were put into the pretreatments weekly, no new microbial growth would occur and attack the cotton, so no activity would register. However the 1g samples would have still contained viable microbes from the growth which occurred during the warm period of incubation.

The FDA assay was found to have been used for various viability tests (see section 4.2 for details). It could have theoretically been possible for enzymes produced by the embryo of *Rosa corymbifera* 'Laxa' to be giving exaggerated results for activity. However it is not believed to be relevant to this study for several reasons. As the FDA assay only had a 30 minute incubation, and the seed coats of *Rosa corymbifera* 'Laxa' are thick, it is highly improbable that any enzymes could leach out in that time (Pritchard, 1997 pers. comm).

The results of the FDA assay on the seeds of *Rosa corymbifera* 'Laxa' confirm this view (table 4.7). FDA activity was very low for untreated, viable seeds (1.1µg fluorescein) and undetectable for dead seed (autoclaved seed). Commercially pretreated seed sampled after 6 weeks into the warm incubation also had a low activity (0.5µg fluorescein). Also taking 1.0g Garotta pretreated seed at week 6, the very most the seed could have contributed to the overall activity for the 1g sample was 12 % of the amount shown in figure 4.8 (11µg fluorescein was detected for Garotta pretreated seed (table 4.7) but the activity for the same week for the whole sample was nearly 80µg fluorescein). This seed has already been shown to have a microbial loading (section 2.2.2) and it is this microbial loading which will have resulted in the FDA hydrolysis.

The orchid seed study conducted by Pritchard (1985) used a much higher FDA concentration on a very permeable embryo. When approached regarding *the Rosa corymbifera* 'Laxa' work he stated that given the nature of the *Rosa corymbifera* 'Laxa' seed, he would not expect it to interfere with the microbial activity results (Pritchard, 1997 personal communication). The experimental findings also suggest this, as the initial

readings at setup showed very little FDA activity (figure 4.8), as did untreated *Rosa corymbifera* 'Laxa' tested directly (table 4.7).

It is also unlikely that enzymes from the seed embryo would interfere with the FDA assay as fluorescein has a high polarity and is therefore poorly transported through cell membranes (Rotman & Papermaster, 1966). Only excess is excreted (Schnürer & Roswall, 1982), and given the poor permeability of the seed coat and the short incubation time, the low level detected could only be from residual microbial activity associated with the seed.

In this study this would suggest that little would leak out through *Rosa corymbifera* 'Laxa' seed, especially in the unsplit state. If the embryo of *Rosa corymbifera* 'Laxa' were to contribute to the fluorescein activity, it would be most detectable once the seed coat had split open. This splitting occurs only with the Garotta pretreated seed, after approximately 4 weeks in the warm. Such seed only gives an activity of 11.6µg fluorescein which has been attributed to the microbes (table 4.7).

This was higher than the commercially pretreated equivalent as the microbes have already been shown to increase in number compared to the commercially pretreated seed (4.1.2). At the equivalent sample time a value of over 80µg was obtained for the 1g sample (figure 4.8) from the Garotta pretreatment, so the seed could have at most contributed only 15% of the activity - although the contribution from the seed would actually be from the microbes closely associated with the seed surface.

It can therefore be concluded that the FDA assay measured the activity of the microbes present in the pretreatments. Any enzymes originating from the seed would have had neglible effect on the FDA readings

4.3 Assessment of microbial population during pretreatment

The previous two sections of this chapter have looked at microbial numbers and microbial activity. Microbial enhancement has resulted from the addition of Garotta to the pretreatment of *Rosa corymbifera* 'Laxa'. However, neither directly relates to what the microbes could be doing to the seed. This section studies the degradation potential of the microbes by assessing their cellulase production.

Objective - to use the cotton strip assay as an assessment of the microbial population producing cellulase, one particularly important enzyme in plant material degradation.

The cotton strip assay was originally developed as a quantitative assessment of fungicide treatments on textiles (Wade, 1947; Barr, 1988). Concern lay in the prevention of cloth decay, especially by fungal attack. Thus a routine test was devised in which strips of cotton textile were buried in tanks of soil (Wade, 1947; Barr, 1988). The loss in tensile strength of the cotton fibres with time (measured using a tensometer) was therefore used as the quantitative measure of the 'rottenness' of the textile under test - and under these circumstances, the effectiveness of the fungicide. Such was the usefulness of the technique that it became a general commercial test under British Standard 2576 (Anon, 1986).

Inherent in the first applications of this test was the great variability encountered due to different cloth types and soils (Schmidt & Ruschmeyer, 1958). Restriction to one cloth type was not sufficient to counter this problem due to different mills using different fibre mixes, thread densities etc. (Sagar, 1988a). The cloth available today fulfils the adaptation or reversal in the original requirements of the test. What initially began as a study on fungicide treatments on cloth, culminated in a need for cloth for field ecology experiments.

Visual observations showed differences in rotting of cloth at different sites (Latter *et al*, 1967) and the assay was quantified by using tensile strength as the measure of cloth decomposition. The Shirley Institute (one of the founders of research on the burial method by the textile industry) produced the 'Shirley Soil Burial Test Fabric' in 1976 (Walton & Allsopp, 1977; Sagar, 1988). It was soon in use for commercial testing and ecological studies, especially as previous supplies of a 'standardised' cloth had run out (Latter & Walton, 1988).

Cellulose constitutes the main structural component of plant cell walls (Allsopp & Seal, 1986). This is true of seeds in particular, the use of fibres from seeds being common place in textile production. Flax and jute are obtained from phloem fibres, sisal and manila from leaf fibres and cotton is made up from seed fibres (Allsopp & Seal, 1986). The relevance of this assay is therefore evident, especially as *Rosa corymbifera* 'Laxa' seed was shown to be made up from cellulose (section 3.1.2).

4.3.1 Materials and Methods

Shirley Soil Burial Test Fabric (BS 2576) was purchased from Shirley Dyeing and Finishing Limited, Cheshire in 10 metre bolts. Strips were cut and frayed to give a final length of 20cm and width of 25mm. The length of the strips was not crucial, however the width must be frayed to exactly the same number of threads for all strips - it is this characteristic, the warp, which is measured. This is done by using the coloured threads as shown in plates 4.1 and 4.2.

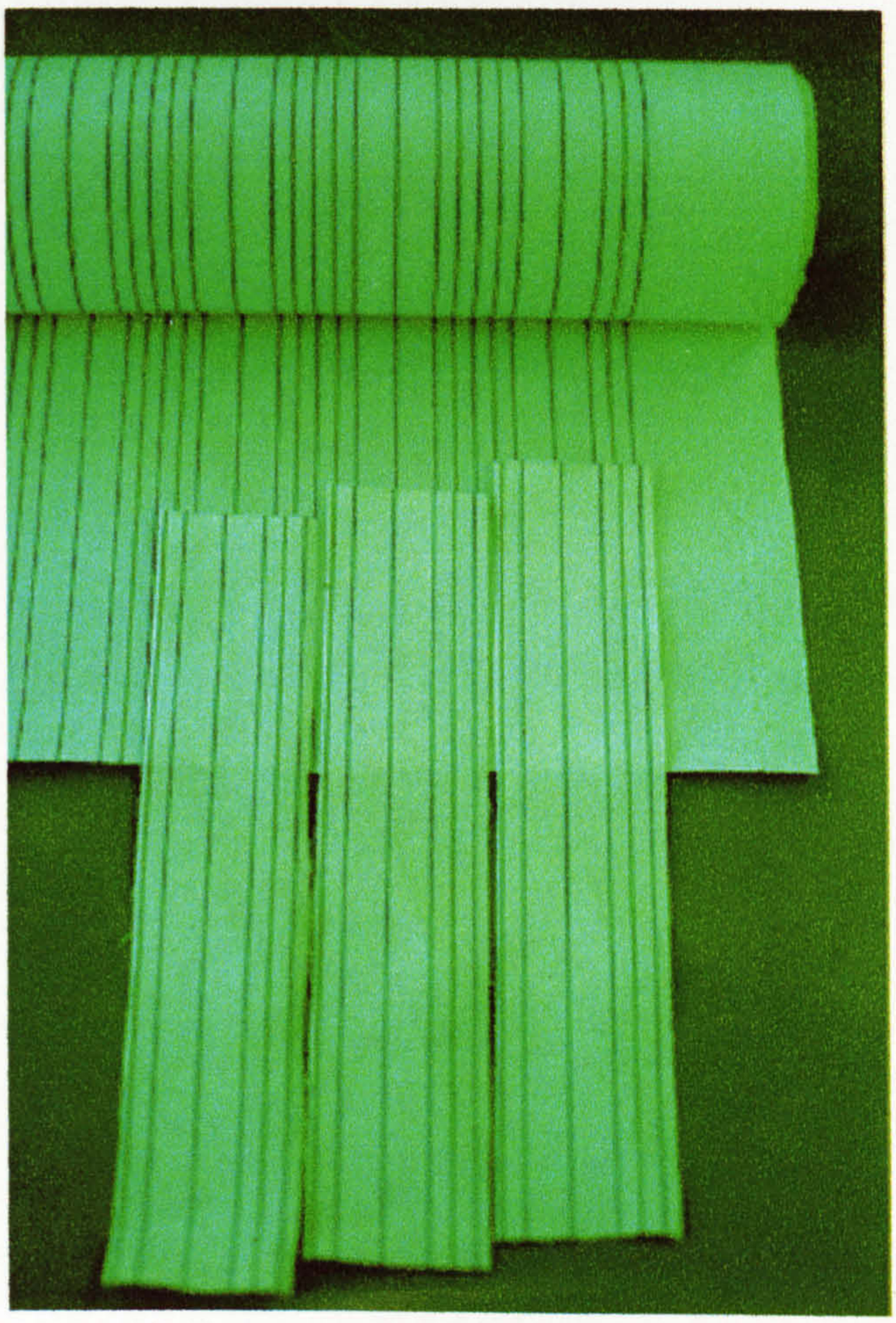


Plate 4.1 The initial cutting of the cotton for the cotton strip assay. The strips are cut to a length of 20cm from the main bolt of cloth.

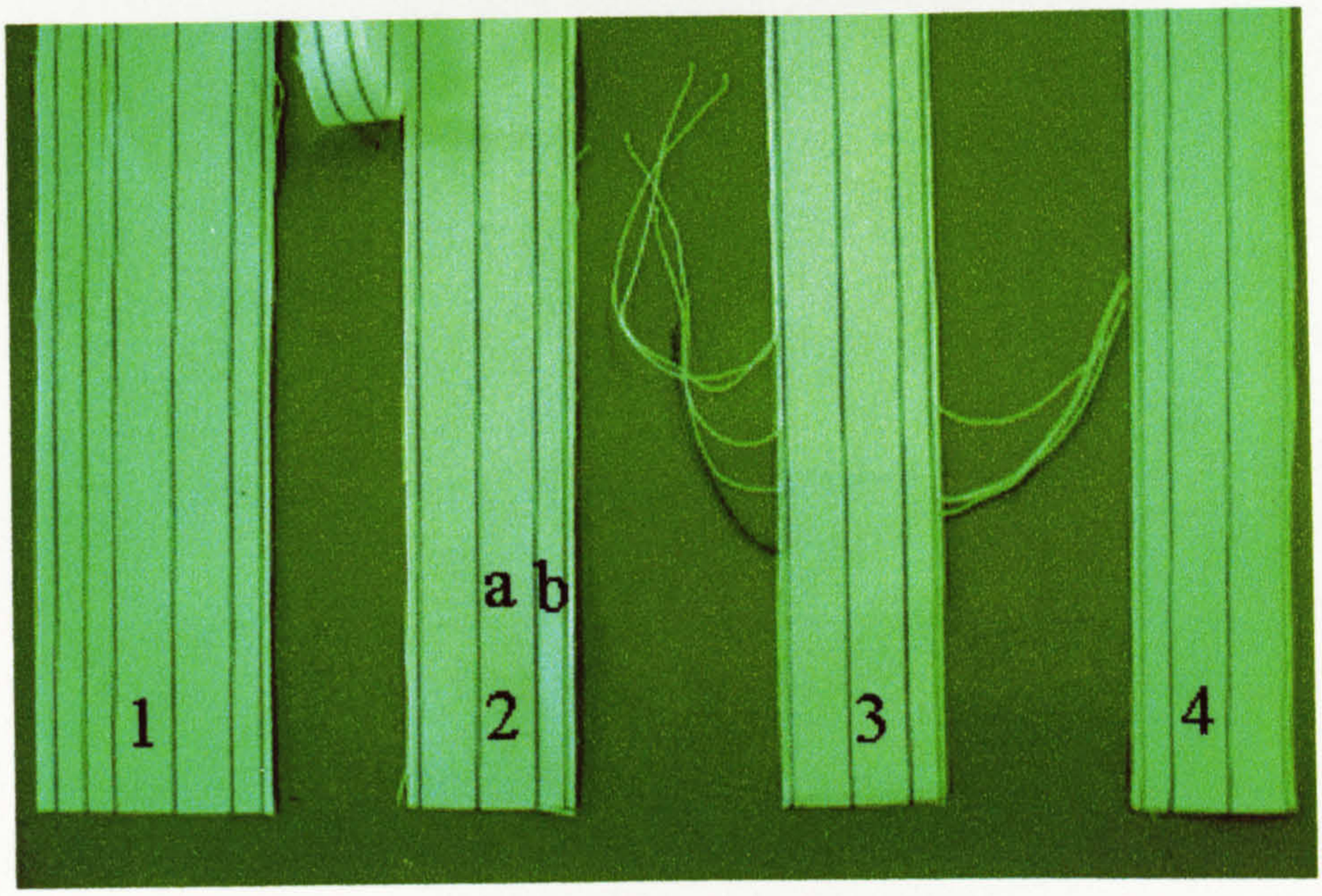


Plate 4.2 The cutting and fraying of cotton strips.

The 20cm strips shown in plate 4.1 are prepared for fraying widthways (1). Cutting along the coloured threads (2) gives a strip approximately 25mm in width. Each section between the coloured threads is either 10mm or 5mm in width (a and b respectively). Each coloured line is made up from two threads. Final fraying pulls away the warp threads until only 1 coloured thread is left on each side (3). The cotton strip is now ready for the assay (4).

Strips were inserted into the pretreatment media using a modified method of Latter and Howson (1977). A blunt wedge shaped stake was used to insert the strips into the vermiculite mix, and backfilled to ensure good contact between cotton and vermiculite (plate 4.3). Approximately 2cm at each end of the strips was left protruding above the surface allowing enough non degraded material to be gripped in the jaws of the tensometer (plates 4.4 and 4.5).

Plate 4.3 Placing the cotton strips into the pretreatment media.

Each cotton strip was carefully placed into the pretreatment medium (in this example in a plastic bag) using a blunt wooden stake.

Plate 4.4 A pretreatment replicate with all five cotton strips in place.

Five cotton strips were put into each replicate of each treatment. The ends were left protruding above the surface to avoid contact with the medium.

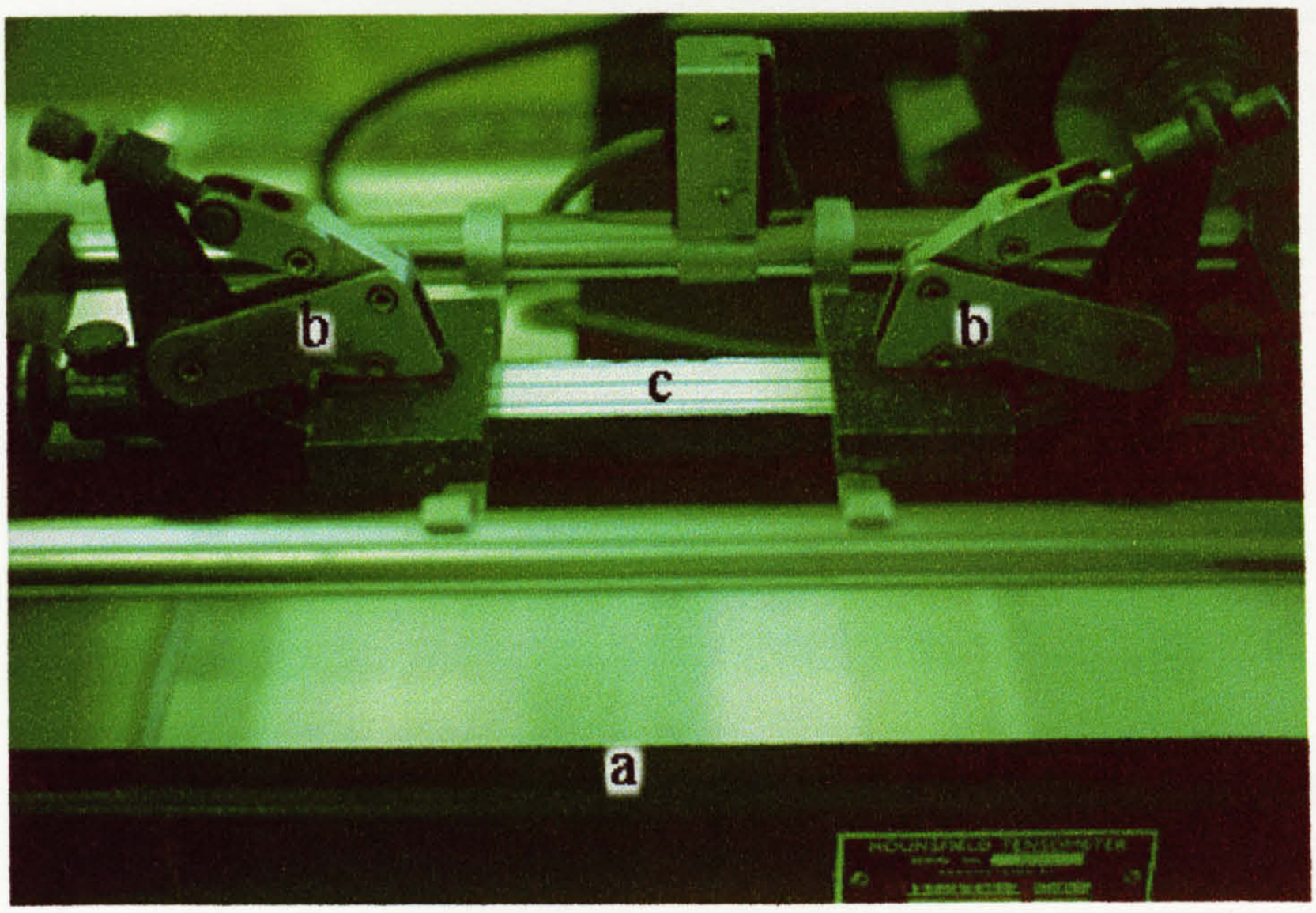


Plate 4.5 A cotton strip ready for testing on the tensometer.

This plate shows a cotton strip (c) placed in the jaws (b) of the tensometer (by the ends which were left protruding above the medium) ready for testing. The platform at the foot of the plate (a) is the base of the tensometer.

4.3.1.1 Comparison of the effect of seed and Garotta on tensile strength

Initial experiments studied the effect on tensile strength of the commercial treatment and Garotta treatment. Also included were a vermiculite only treatment and a vermiculite with Garotta treatment (the amount of Garotta added was the same as was added in the Garotta treatment, 6g). Each treatment was triplicated and 5 cotton strips placed weekly in each. Data was collected for the warm period of the pretreatment and after one week in the cold. The results are presented in section 4.3.2.1.

Control strips (untreated) were analysed once they had been soaked with water and wrung to remove excess. Their tensile strength was then measured by clamping each end in a tensometer (Hounslow tensometer, 600N beam) and stretching until the strip snapped (plate 4.6). A value was recorded from the scale on the beam, the two halves removed and the process repeated for the 5 replicates. Treated strips were similarly processed, being washed then processed.

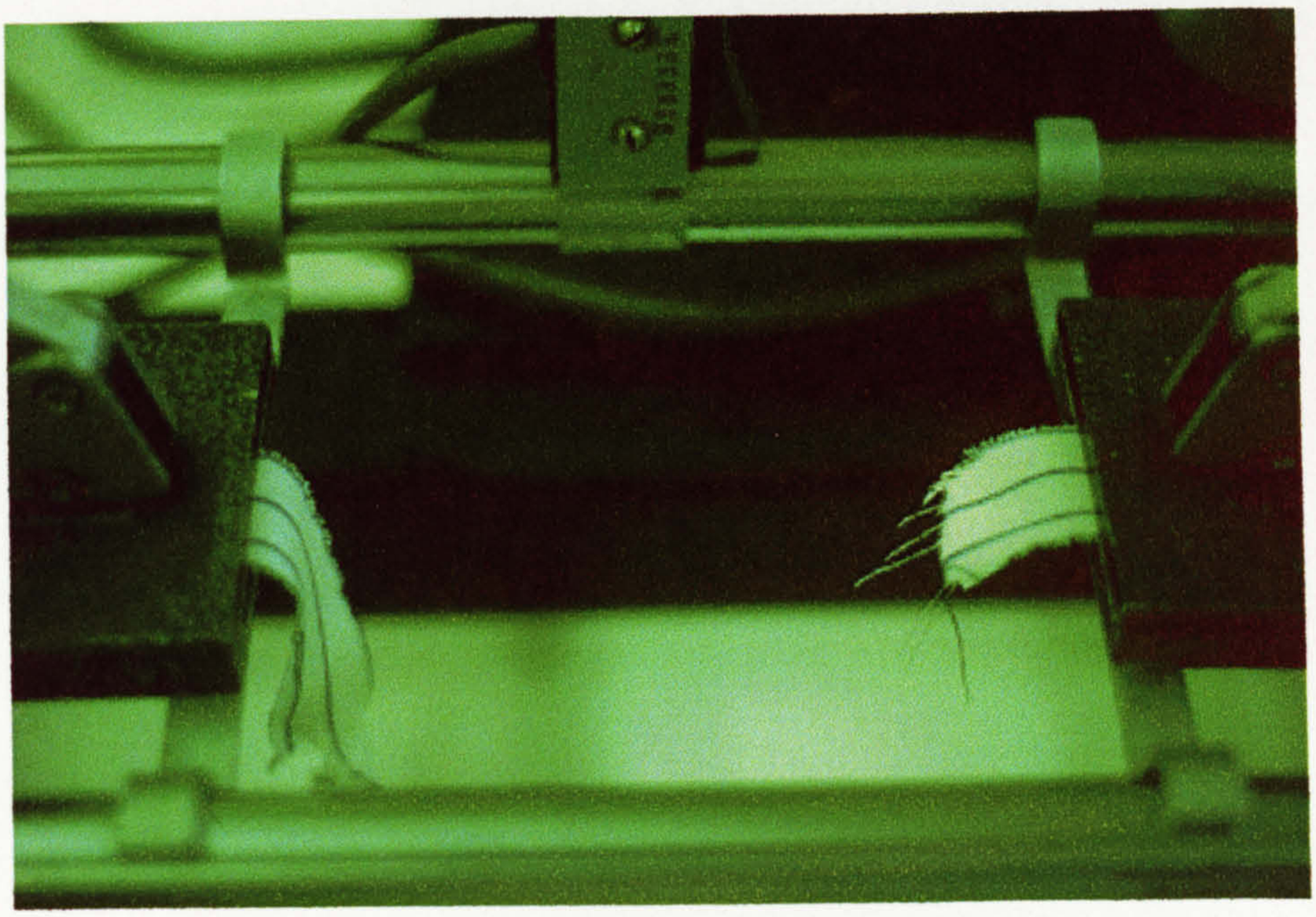


Plate 4.6 A cotton strip following testing.
The cotton strips were stretched until they snapped. The reading was then taken from the beam on the tensometer giving the tensile strength of the cotton strip in newtons.

4.3.1.2 Comparison of the commercial, 1.0g and 0.5g Garotta pretreatments

Following on from the initial experiments, three treatments were monitored for tensile strength loss; commercial, 1.0g Garotta and 0.5g Garotta - as were used in the initial FDA work (section 4.1.1). Cotton strip data were collected for the warm period of incubation only in this experiment. The results for this experiment are presented in section 4.3.2.2.

Five strips were placed in each treatment. After seven days incubation in the mix (at either 25°C or 4°C depending on stage of treatment), strips were removed and loose material shaken off. Five fresh strips were then inserted into the containers and incubation continued. The removed strips were usually frozen in aluminium foil until sufficient numbers were available to test. Freezing at -20°C reduced microbial activity to a minimum, thus no further reduction in strength would occur.

4.3.2 Results

4.3.2.1 Comparison of the effect of seed and Garotta on tensile strength

The loss in tensile strength is expressed as percentage loss (compared with untreated control strips). The results are represented graphically below (figures 4.10, 4.11 and 4.12).

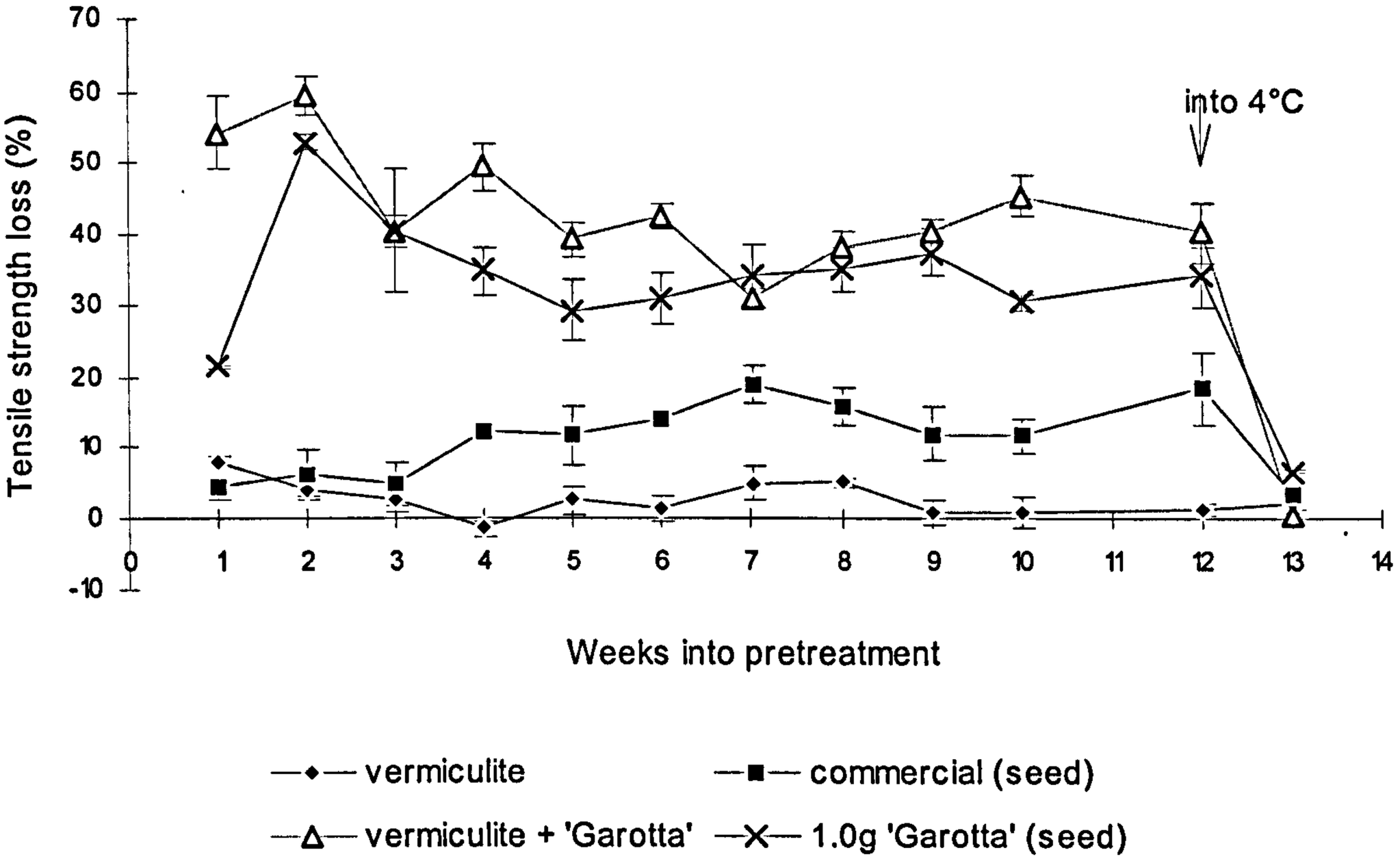


Figure 4.10 Loss in tensile strength of cotton strips for the first 13 weeks of the commercial, 1.0g Garotta, vermiculite only and vermiculite with Garotta pretreatments of *Rosa corymbifera* 'Laxa'. Each data point represents the average of the means of three replicates (5 cotton strips in each) from each treatment. Standard error bars are shown for each point (where absent, the SE is too small to register).

Table 4.10 Levels of significance between pretreatments for the cotton strip assay results (shown graphically above). Each combination of treatments was analysed separately using one way analysis of variance.

	Week											
	1	2	3	4	5	6	7	8	9	11	12	13
Treatments compared												
vermiculite (- seed) and commercial (+ seed)	ns	ns	ns	0.1%	1%	0.1%	0.1%	1%	0.1%	0.1%	0.1%	ns
vermiculite (- seed) and 1g Garotta (- seed)	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	ns
vermiculite (- seed) and 1g Garotta (+ seed)	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	5%
commercial (+ seed) and 1g Garotta (- seed)	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	5%
commercial (+ seed) and 1g Garotta (+ seed)	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	ns
1g Garotta (- seed) and 1g Garotta (+ seed)	0.1%	5%	ns	0.1%	1%	0.1%	ns	ns	ns	0.1%	1%	0.1%

The treatments with Garotta show a much greater loss of tensile strength of the cotton strips than either of the other treatments. The loss in strength follows a similar pattern for both Garotta treatments, with a slightly greater loss in the treatment without seed. The commercial treatment has a consistently greater loss (except after week one) than the vermiculite on its own. The loss in strength of cotton strips in all four treatments reverted to almost zero once the treatments were put in to the cold (week 13).

There was no statistical difference within treatments following a one way analysis of variance for all four treatment types, except in weeks 6 and 11 of the vermiculite (- seed), weeks 3, 5 and 7 of the 1.0g Garotta (- seed) and week 1 of the 1.0g Garotta (+ seed) treatments.

Table 4.10 summarises the statistical data for comparison between the four treatments. Whilst there was significant difference between almost all treatments at all time points, of particular interest is the fact that there was a very highly significant difference between the commercial and 1.0g Garotta treatments every week until week 13. There was no significant difference in week 13, this was after the first week of cold incubation, when microbial activity has already been shown to diminish (section 4.2.3).

In all but one case the significant difference between treatments diminished on transfer of the treatments into the cold, in three cases to no significant difference.

4.3.2.2 Comparison of the commercial, 1.0g and 0.5g Garotta pretreatments

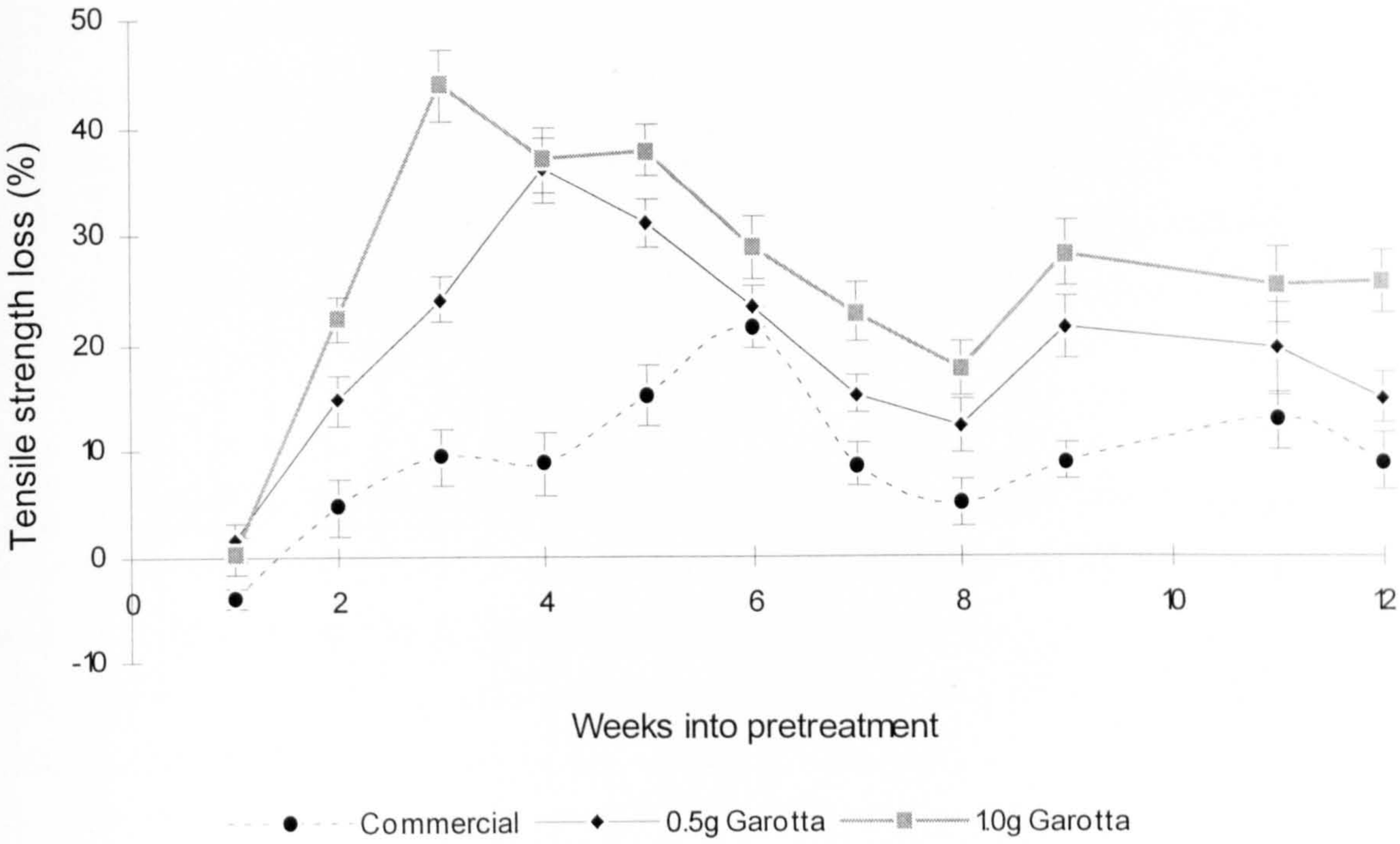


Figure 4.11 Loss in tensile strength of cotton strips during the warm pretreatment of *Rosa corymbifera* 'Laxa'. Each data point represents the average of the means of three replicates (5 cotton strips in each) from each, exposed for 7 days to the pretreatment. Standard error bars are shown for each point.

After only one week incubation at 25°C the Garotta pretreatment had already shown 6% loss in tensile strength of the cotton strips. The cotton strips in the commercial pretreatment had actually gained slight strength compared to untreated control, but this was not a new phenomenon as previous studies had experienced similar results (Smith & Maw, 1988). The 0.5g Garotta pretreatment hit a peak loss in tensile strength after 4 weeks (36%) and the 1.0g Garotta after 3 weeks (44%). The commercial pretreatment had an overall much lower loss in tensile strength of the strips for the entire warm period, reaching a maximum loss after 6 weeks (21%).

Table 4.11 Levels of significance comparing the cotton strip results of the commercial pretreatment against the 1.0g Garotta pretreatment.

	Week										
	1	2	3	4	5	6	7	8	9	11	12
commercial and 1g Garotta	ns	0.1%	0.1%	0.1%	0.1%	0.1%	5%	0.1%	0.1%	1%	0.1%

All three pretreatments followed a similar trend in tensile strength loss. Initially values were low, but tensile strength loss increased to a maximum before slowly tailing off. Each pretreatment has a peak of tensile strength loss. The greatest loss in strength in all the pretreatments occurred in the quickest time with the Garotta pretreatment. Conversely, the least tensile strength loss of any pretreatment was after the longest time and was in the commercial pretreatment. The peak tensile strength loss of the 0.5g Garotta pretreatment occurred midway between the other two pretreatments, both in value and time into the pretreatment.

There was no significant difference between replicates (one way analysis of variance) within each pretreatment with the exception of week 3 in the commercial pretreatment (see appendix 4.5 for summary table). Comparing the commercial pretreatment with the 1.0g Garotta pretreatment resulted in significant differences from week 2 until week 12. No significant difference was found for the first week (see table 4.11).

As there was a marked difference in the tensile strength loss between the commercial and 1.0g Garotta pretreatments, experiments which were conducted after this used these two pretreatments only. The 0.5g Garotta treatment was discontinued.

The graph in figure 4.12 shows the results for the warm and cold incubation of both the commercial and Garotta pretreatments of *Rosa corymbifera* 'Laxa'. The difference in tensile strength loss between the two treatments is vast. The pattern of tensile strength loss is the same as that found in the previous experiments (figures 4.11 and 4.12), although exact figures do vary. The maximum loss of 49% was reached after only 2 weeks for the Garotta pretreatment and only 14% after 4 weeks for the commercial pretreatment.

The effect on tensile strength loss was extremely marked on placing the pretreatments into the cold incubation period (4°C, after 12 weeks). Both treatments responded immediately by a reduction to a negligible loss in strength i.e. cotton strips were as strong as untreated control strips.

Analysis of the results found no significant difference within treatments (one way analysis of variance) (i.e. between replicates) except for weeks 12 and 22 in the commercial treatment and weeks 12 and 22 in the 1.0g Garotta treatment (see appendix 4.5 for summary table). Significance between the two treatments is shown below.

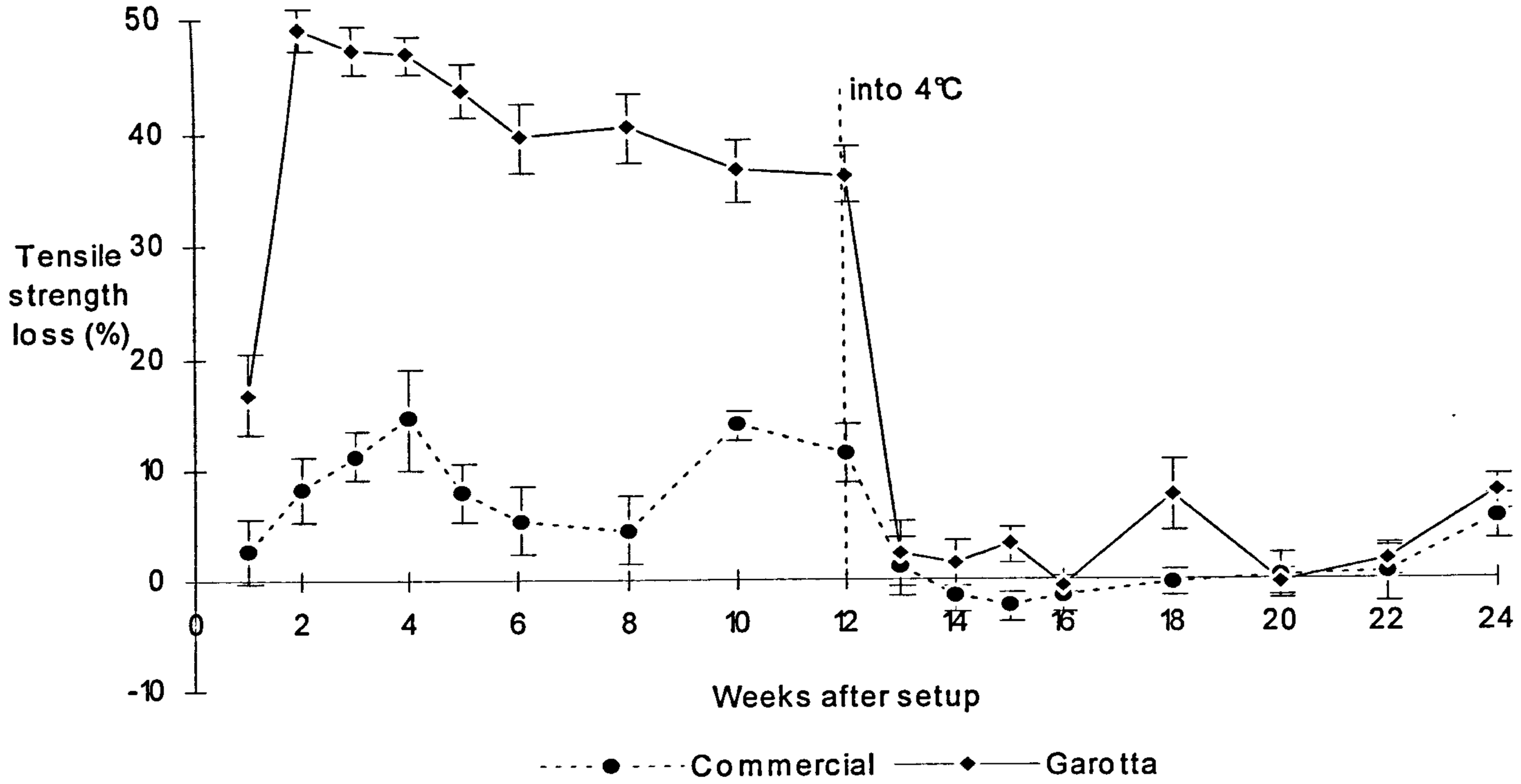


Figure 4.12 Loss in tensile strength of cotton strips during the 24 week pretreatment of *Rosa corymbifera* 'Laxa'.
Each point is the combination of two replicates (i.e. the mean of 10 strips), exposed for 7 days to the treatment. Standard error bars are shown for each point.

Table 4.12 Levels of significance comparing the cotton strip results of the commercial and the 1.0g Garotta pretreatments for the 24 week period.

	Week																
	1	2	3	4	5	6	8	10	12	13	14	15	16	18	20	22	24
commercial and 1g Garotta	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	ns	ns	5%	ns	ns	ns

4.3.3 Discussion

In all the experiments cotton strips tested from the commercial pretreatment showed some tensile strength loss (figures 4.10, 4.11 and 4.12), indicating that the microbial activity present does produce cellulases and can cause limited degradation. When the seed is absent from the commercial treatment (i.e. vermiculite only), no loss in tensile strength of the cotton strips is found (figure 4.10).

The Garotta treatment and Garotta with no seed treatment showed similar higher levels of tensile strength loss. The growth of the microbes in both treatments (i.e. with and without seed) was boosted by the nutrients provided by the Garotta. These microbes would be attacking the seed as well as the cotton strips. The microbes in the Garotta only treatment had no such seed to 'distract' them from the cotton, and thus with the enhanced nutrient levels would attack the only carbon source available - the cotton strips. Previous studies using cotton strips have found that the rotting of the strips depended upon the amount of nitrogen available (Howard, 1988).

The microbes present are capable of producing enzymes which cause the loss in tensile strength of the cotton. It is likely that the commercial and vermiculite only treatments have little or no degradation of cotton strips as available nitrogen is limiting. Carbon is not limiting as it is present in the cotton, however this results in a very high C/N ratio which does not favour microbial growth.

Treatments which have had Garotta added are not so limited, as Garotta has a high level of available nitrogen thus reducing the C/N ratio. It has been stated that phosphorus as well as ntirogen may be a limiting factor for microbial growth (Campbell, 1985). The treatments without Garotta have no obvious phosphorus source except any contained in the seed, whereas the addition of Garotta raises this level, making it no longer limiting.

4.4 Microbes on seeds during pretreatment

Objective - to show that microbes are present on the seeds during the commercial and Garotta pretreatment using scanning electron microscopy.

4.4.1 Materials and Methods

Seeds were sampled at four different times during the pretreatments, at setup, after 3 weeks, 12 weeks and 14 weeks (i.e. 2 weeks into the cold period). Samples of seed taken at setup were also subjected to a humid environment (see section 3.3.1) to encourage growth of any microbes which may be present. The results show 4 seeds from each sample (3 for the setup, 12 and 14 week sampling), each seed having been chosen as a 'typical' representation of the larger sample. Seed was prepared for the scanning electron microscope and viewed as described in the materials and methods of chapter 3 (section 3.1.1).

4.4.2 Results

The plates below were taken of seed, sampled as described above, from the different pretreatments at the four different times during pretreatment as described above (section 4.1.1), and summarised below. The examples included represent the 'typical' seeds found during these studies.

There are four groups of plates presented in this section. The first is of seed taken at setup, i.e. prior to being placed into the pretreatment mix. The second and third groups of plates are of seed sampled after 3 and 12 weeks respectively, from both the commercial and Garotta pretreatments. The final group of plates is of seed sampled in week 14, i.e. 2 weeks into the cold period of pretreatment.

The results section is divided into four subsections, corresponding to the four time periods sampled. Each subsection then has *Rosa corymbifera* 'Laxa' seed sampled from the commercial pretreatment and Garotta pretreatment. The four subsections are as follows;

4.4.2.1 Seed sampled at setup

4.4.2.2 Seed sampled after 3 weeks (peak microbial activity)

4.4.2.3 Seed sampled at 12 weeks (end of warm period)

4.4.2.4 Seed sampled at 14 weeks (2 weeks into cold period)

4.4.2.1 Seed sampled at setup

Plates 4.7 to 4.12 are of seed sampled before it was placed in any pretreatment.

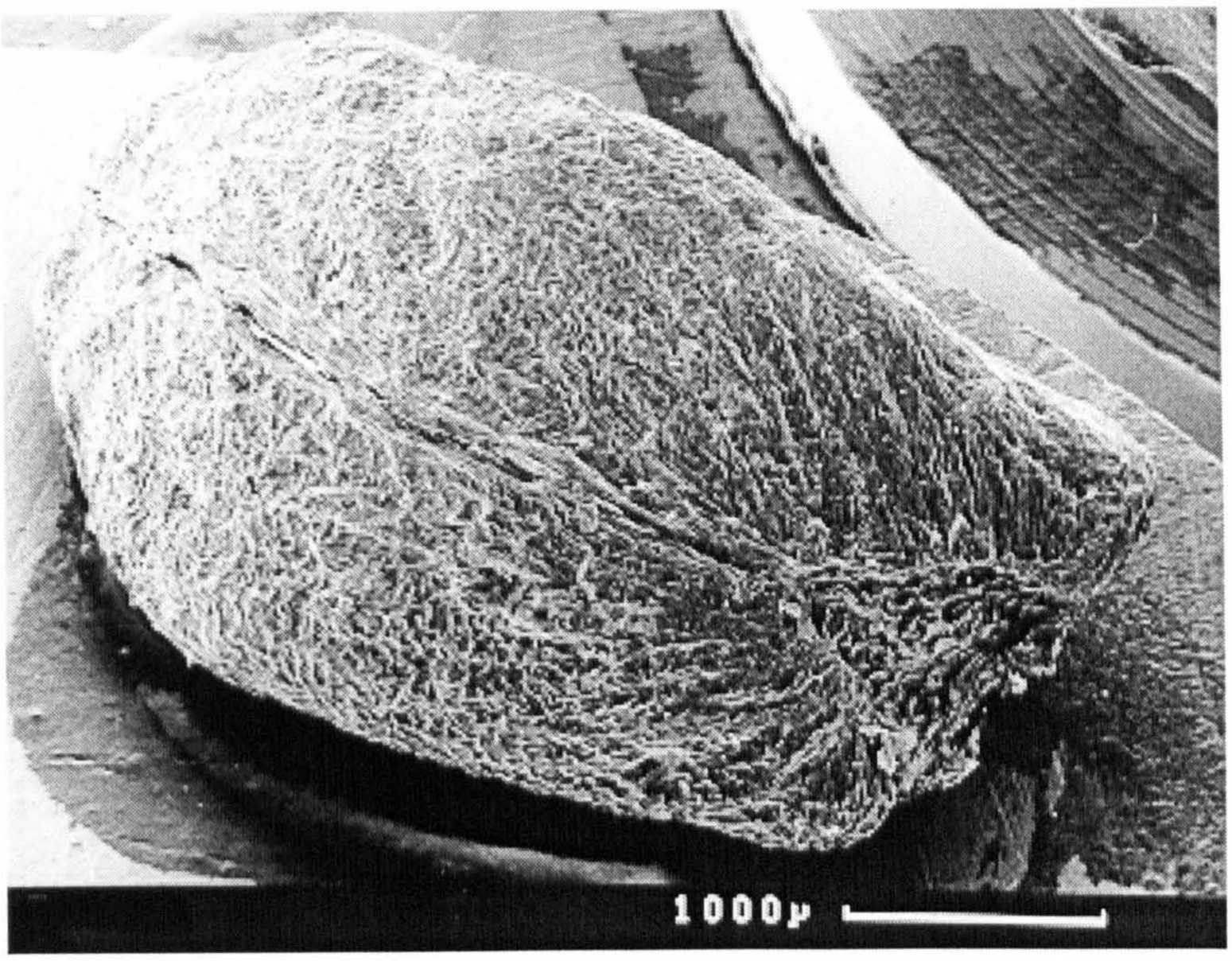


Plate 4.7 The surface of the *Rosa corymbifera* 'Laxa' seed.
The surface clean and shows no sign of microbes. The irregular, rough appearance of the seed coat is normal for this species (the anatomy section (plates 3.6 - 3.9) also show this).

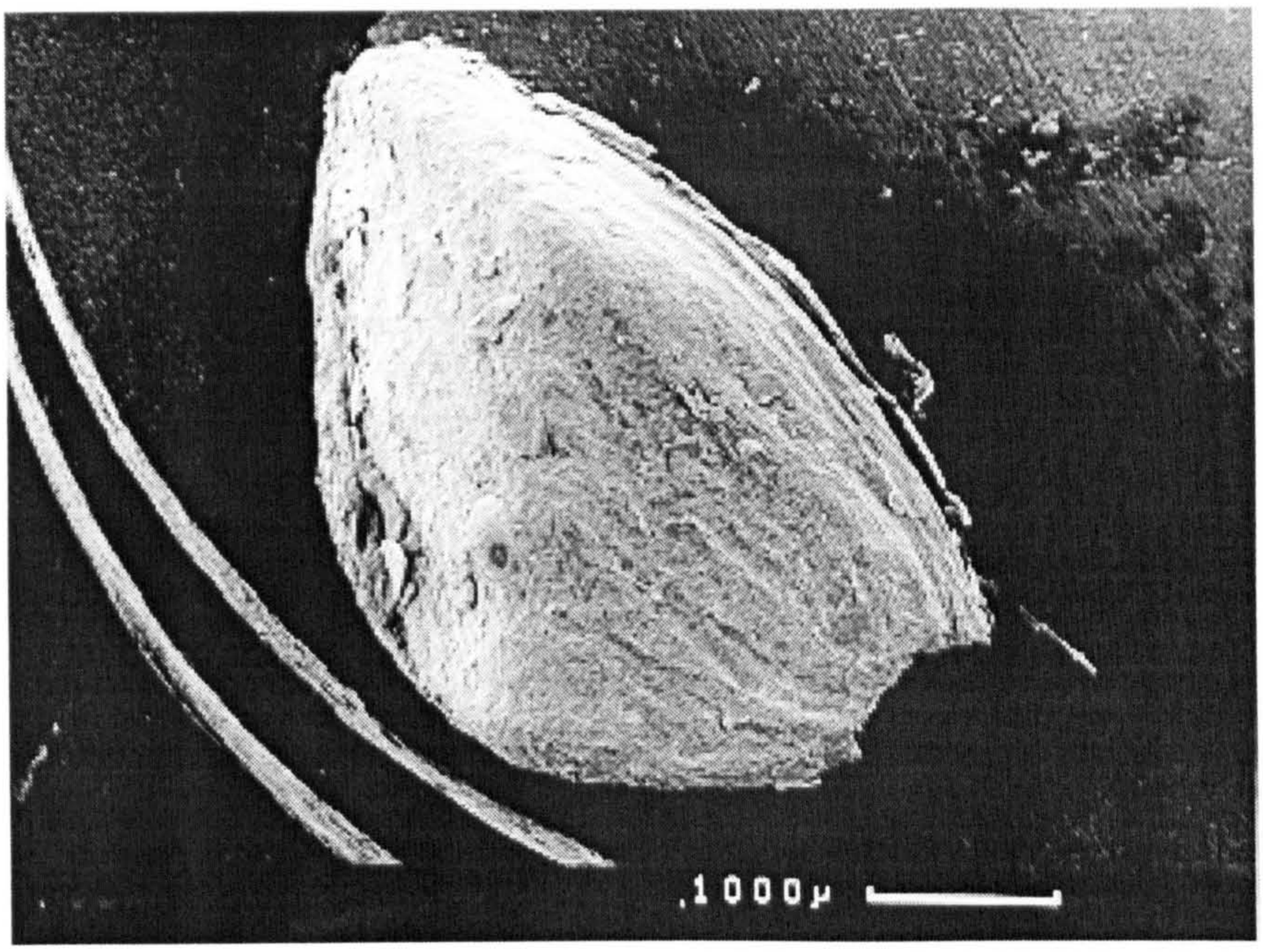


Plate 4.8 This seed also has a clean, microbe free surface.

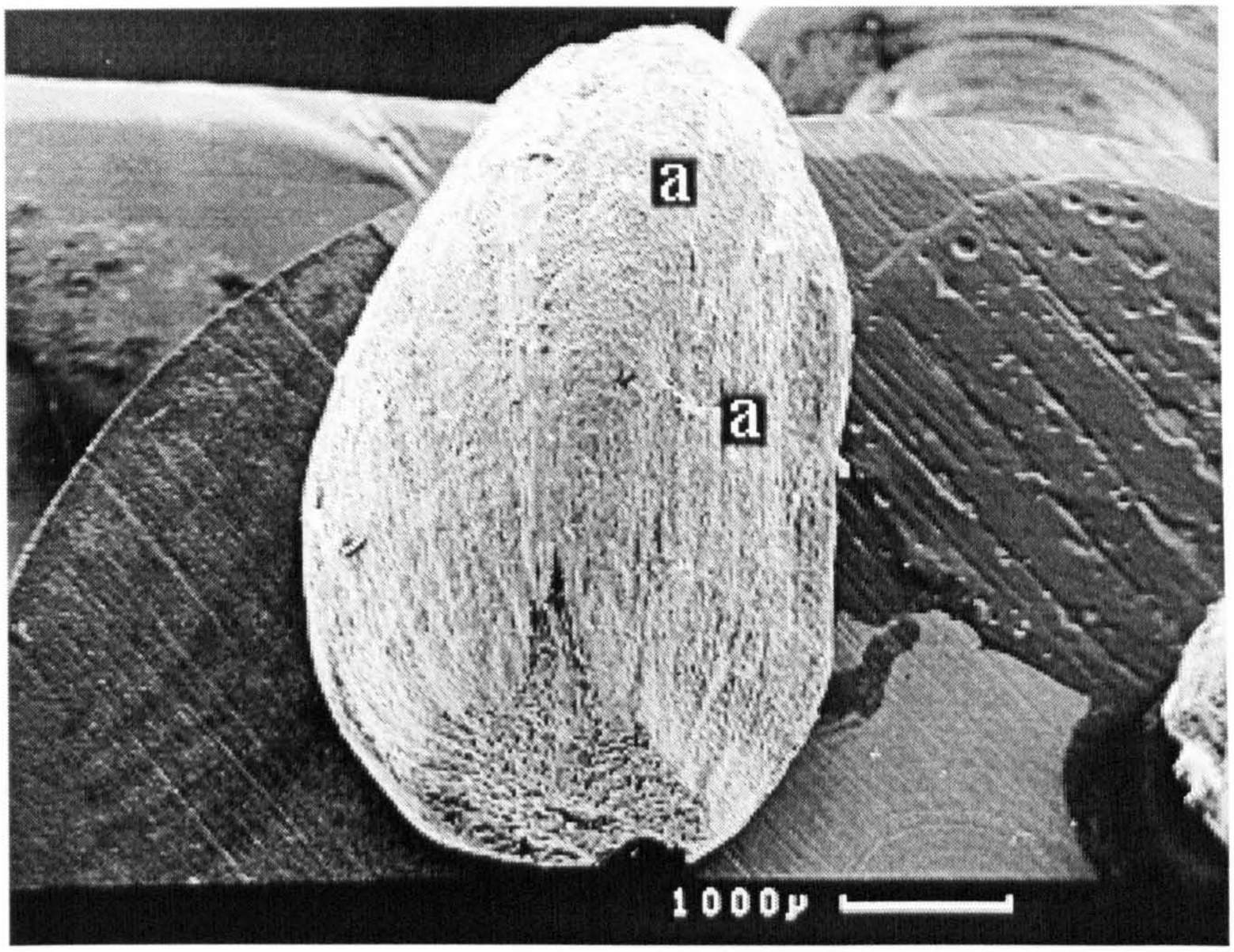


Plate 4.9 The majority of the surface of this seed appears clean.
However on closer examination there are a few small areas with microbes present (a).

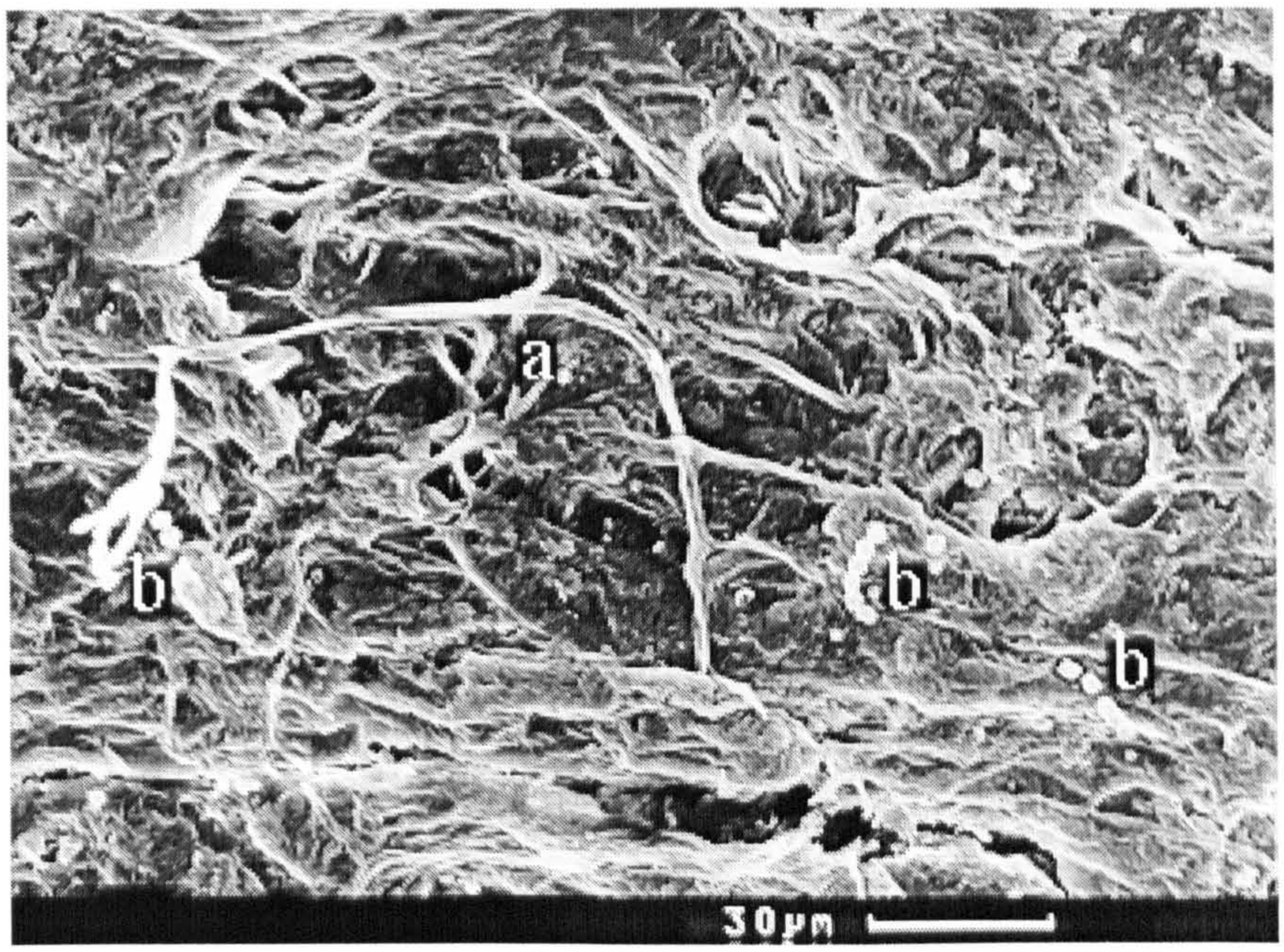


Plate 4.10 Closer inspection of the seed coat from plate 4.9.
This shows some microbial structures, a fungal hyphae (a) and a few spores (b).

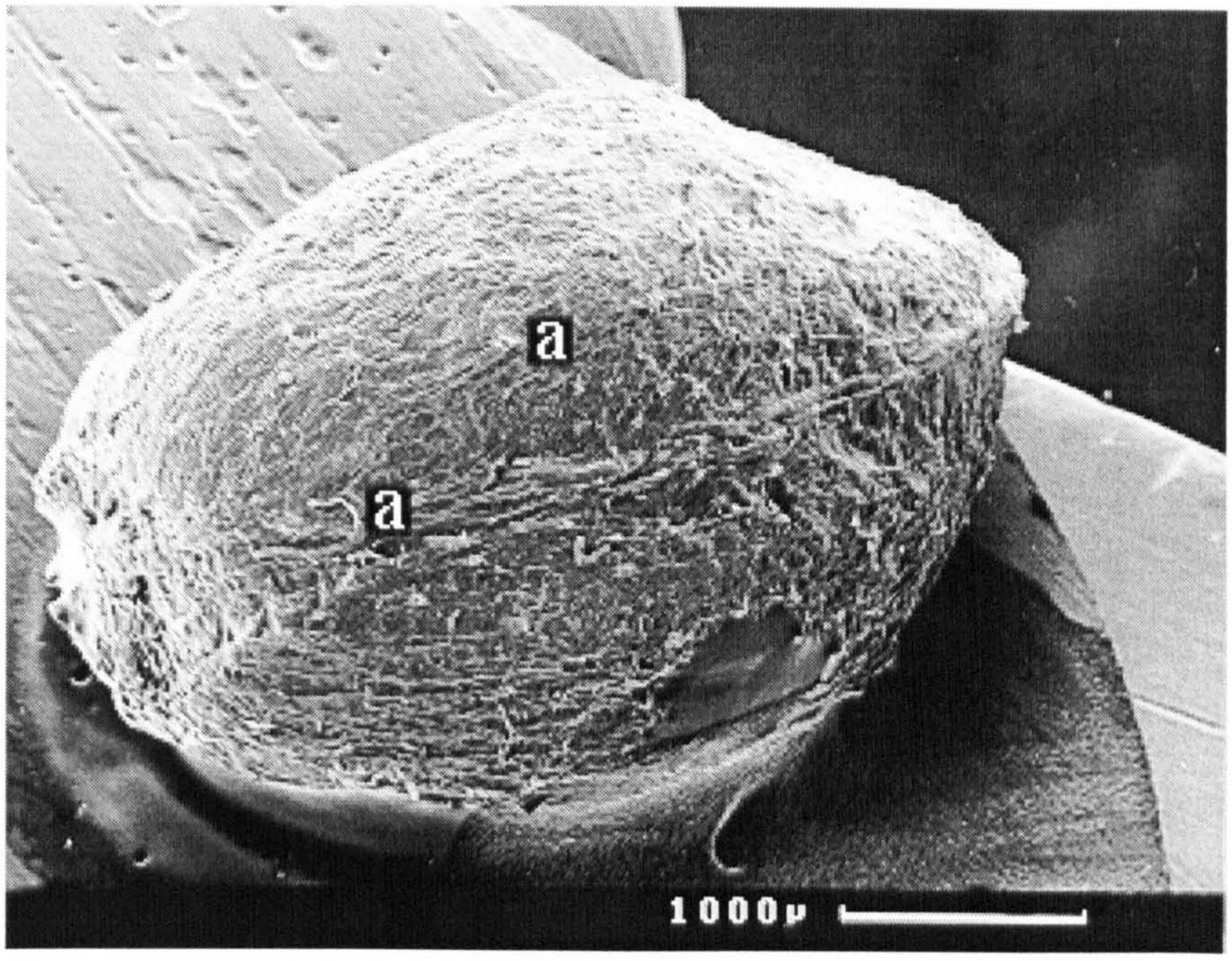


Plate 4.11 Another seed with a relatively clean seed coat.
Although as with the seed in plate 4.9, some microbial structures can be seen (a).

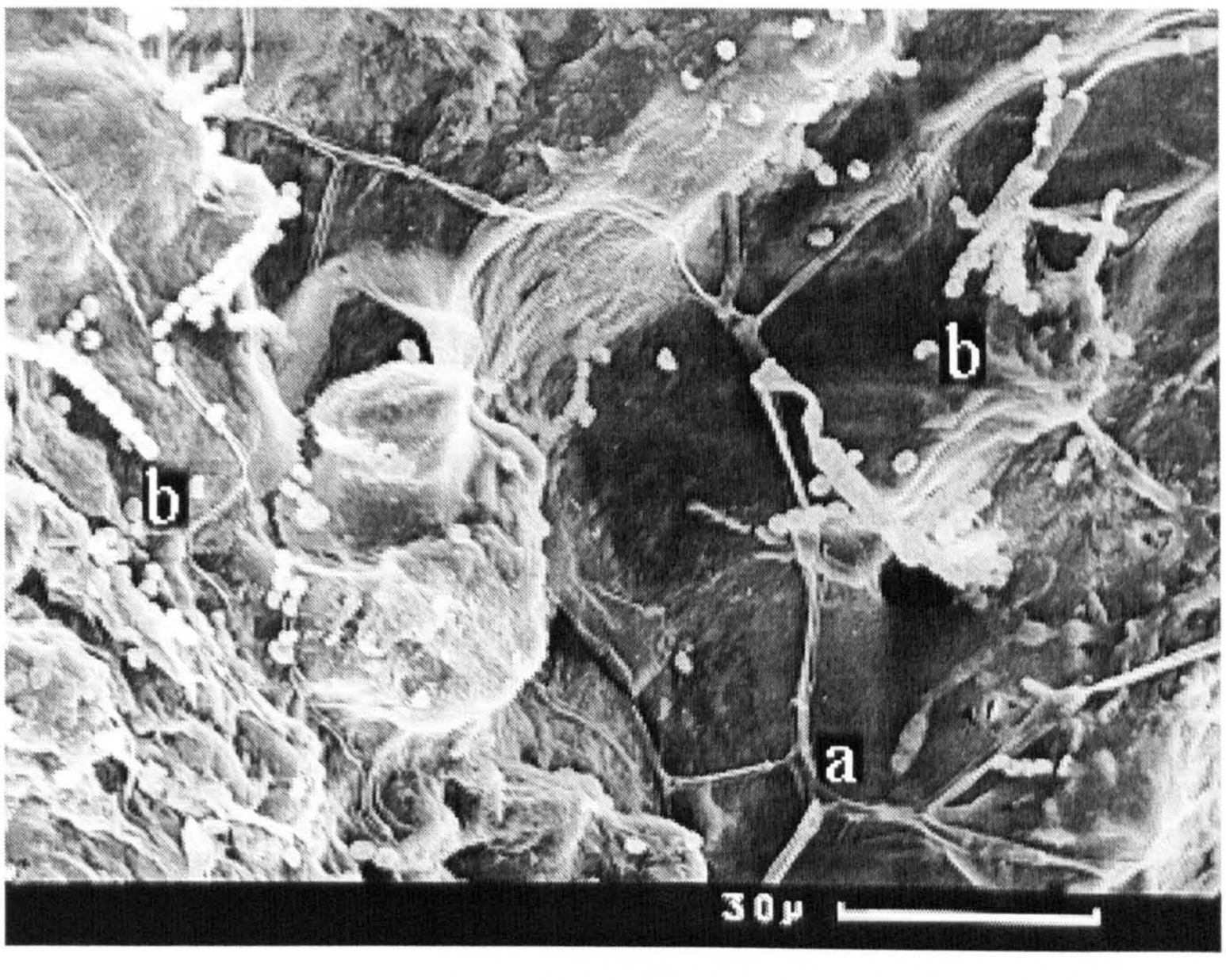


Plate 4.12 Enlarging one of the areas from plate 4.11.
Microbial structures located on the surface reveals evidence of fungal hyphae (a) and spores (b).

Untreated *Rosa corymbifera* 'Laxa' seed having been subjected to moist conditions is shown in plates 4.13 to 4.18 (previously described in section 3.3.2).

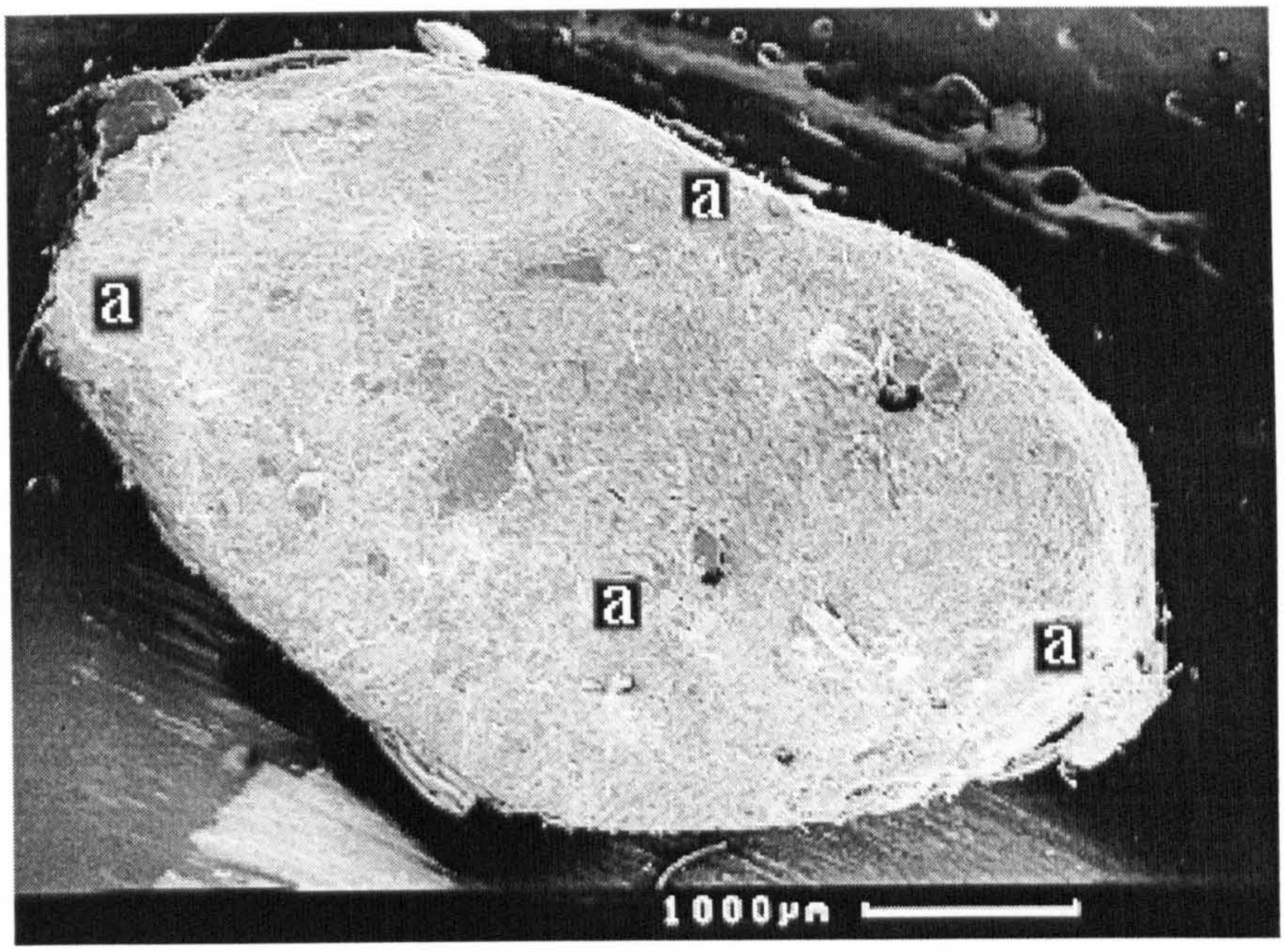


Plate 4.13 The seed coat has abundant microbial structures such as hyphae and spores across the whole surface (a).

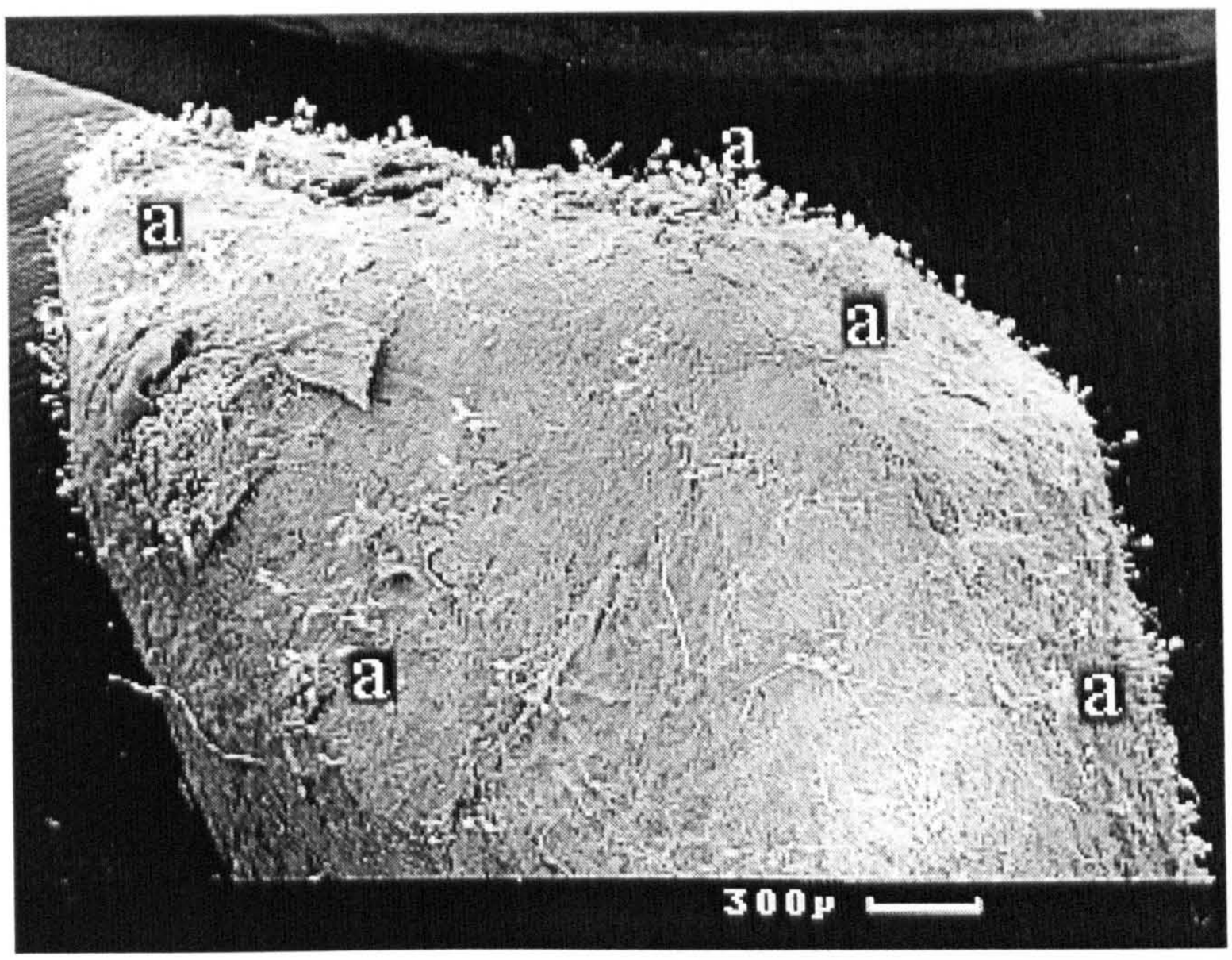


Plate 4.14 The effect of incubation in a moist environment is easily seen in this plate. The seed coat is carpeted with fungal hyphae, spores and fruiting bodies. These are particularly evident on the top edge of the seed (a).

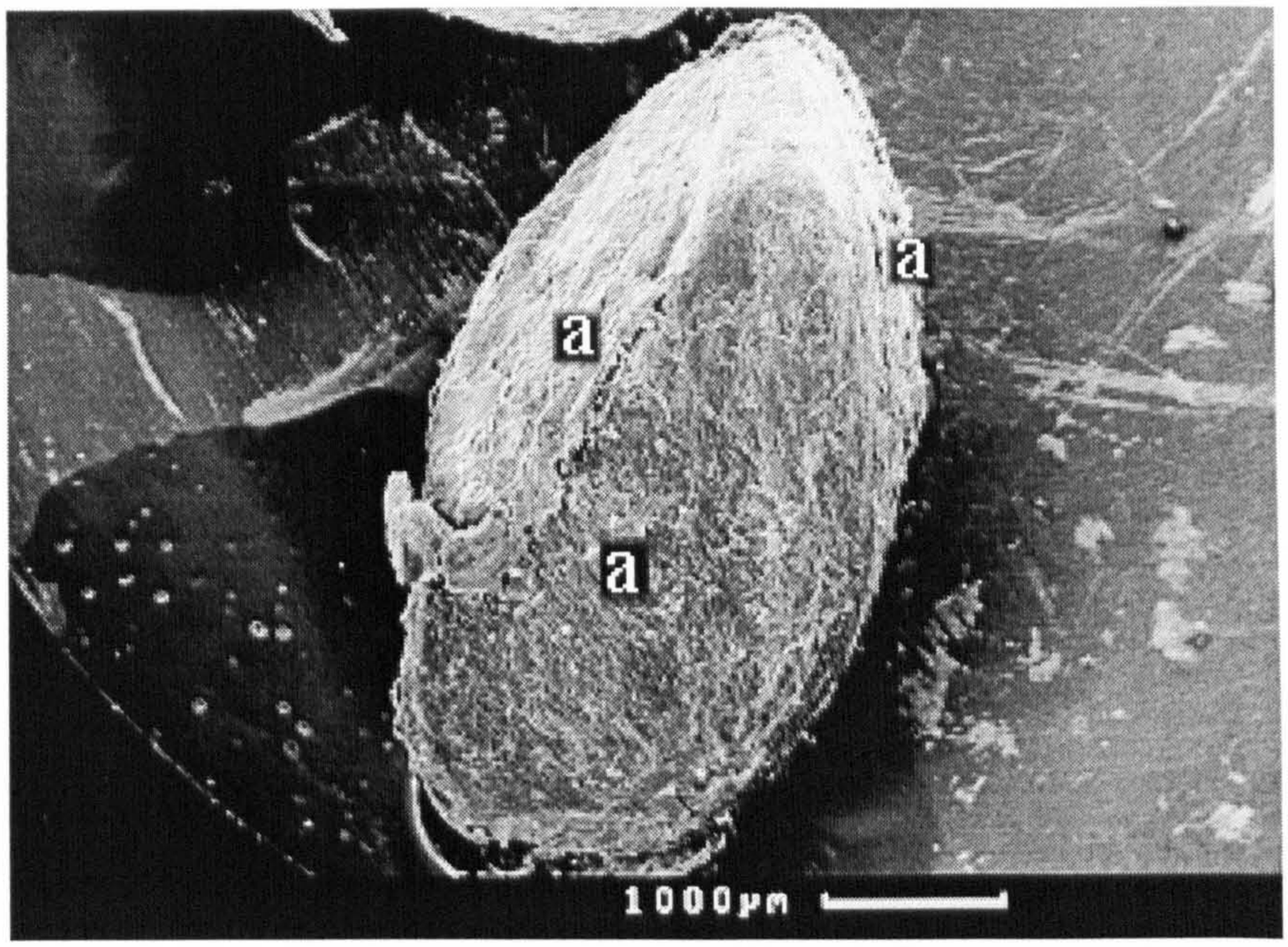


Plate 4.15 An intact *Rosa corymbifera* 'Laxa' seed which also shows a well covered seed coat with microbes (a).

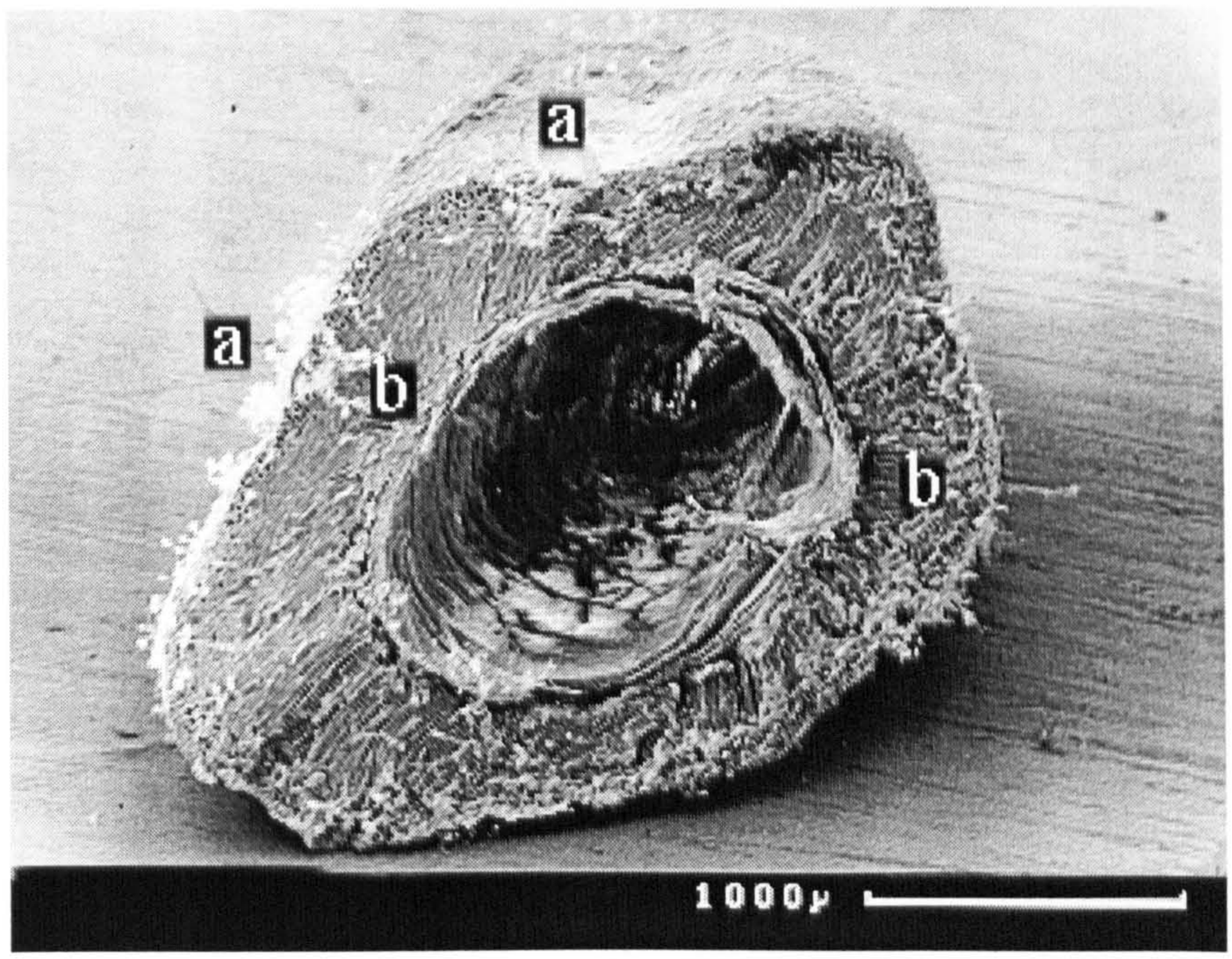


Plate 4.16 A cross section cut prior to incubation under moist conditions.
This shows clearly the mass of microbial structures. These are concentrated on the outer surface of the seed coat (a), where the microbes would originate from, but has slowly encroached onto the cut surface of the seed (b).

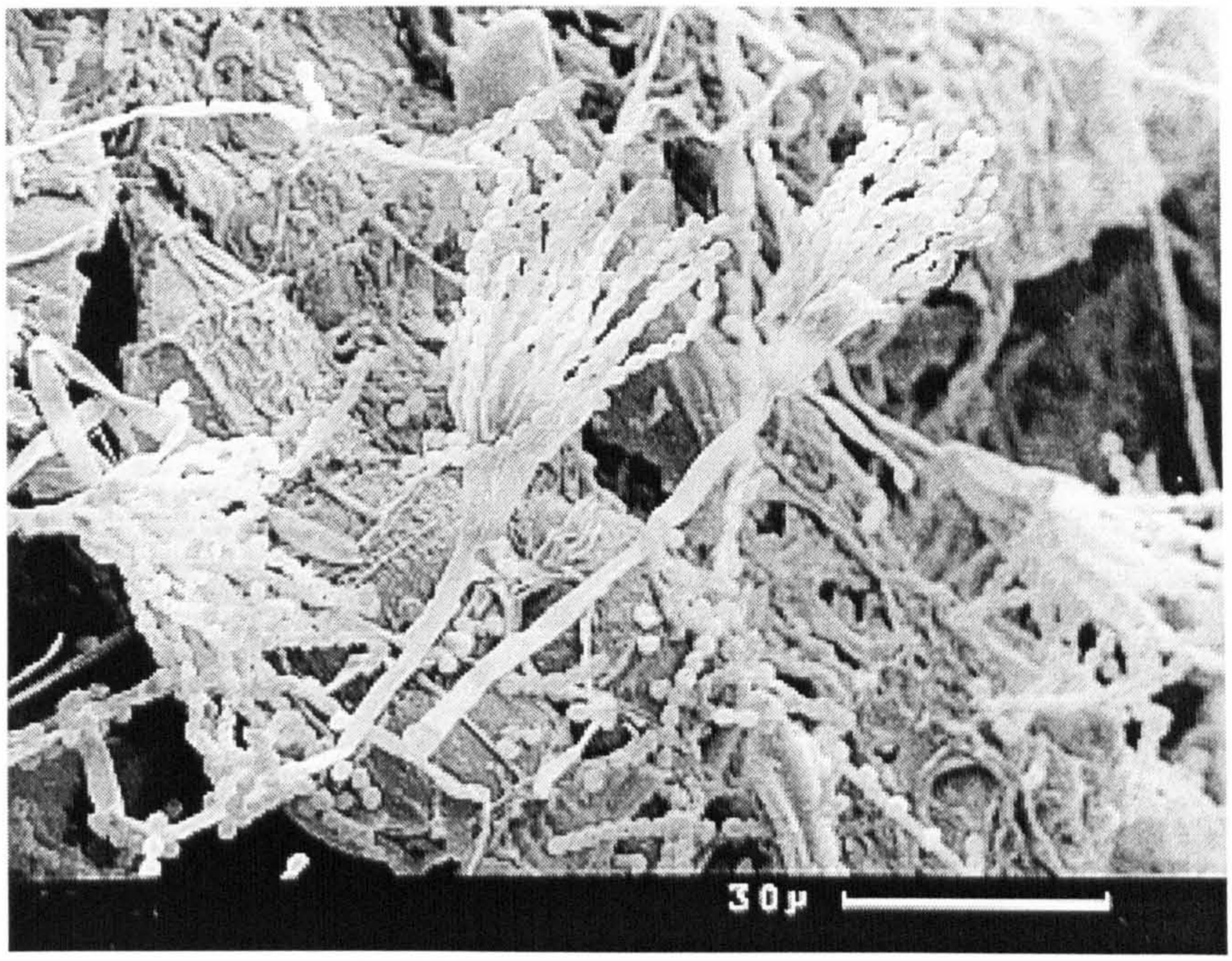


Plate 4.17 A highly magnified plate of one of the areas of growth showing clearly the fungal hyphae and chains of spores. The anatomy indicates this to be a *Penicillium* species.

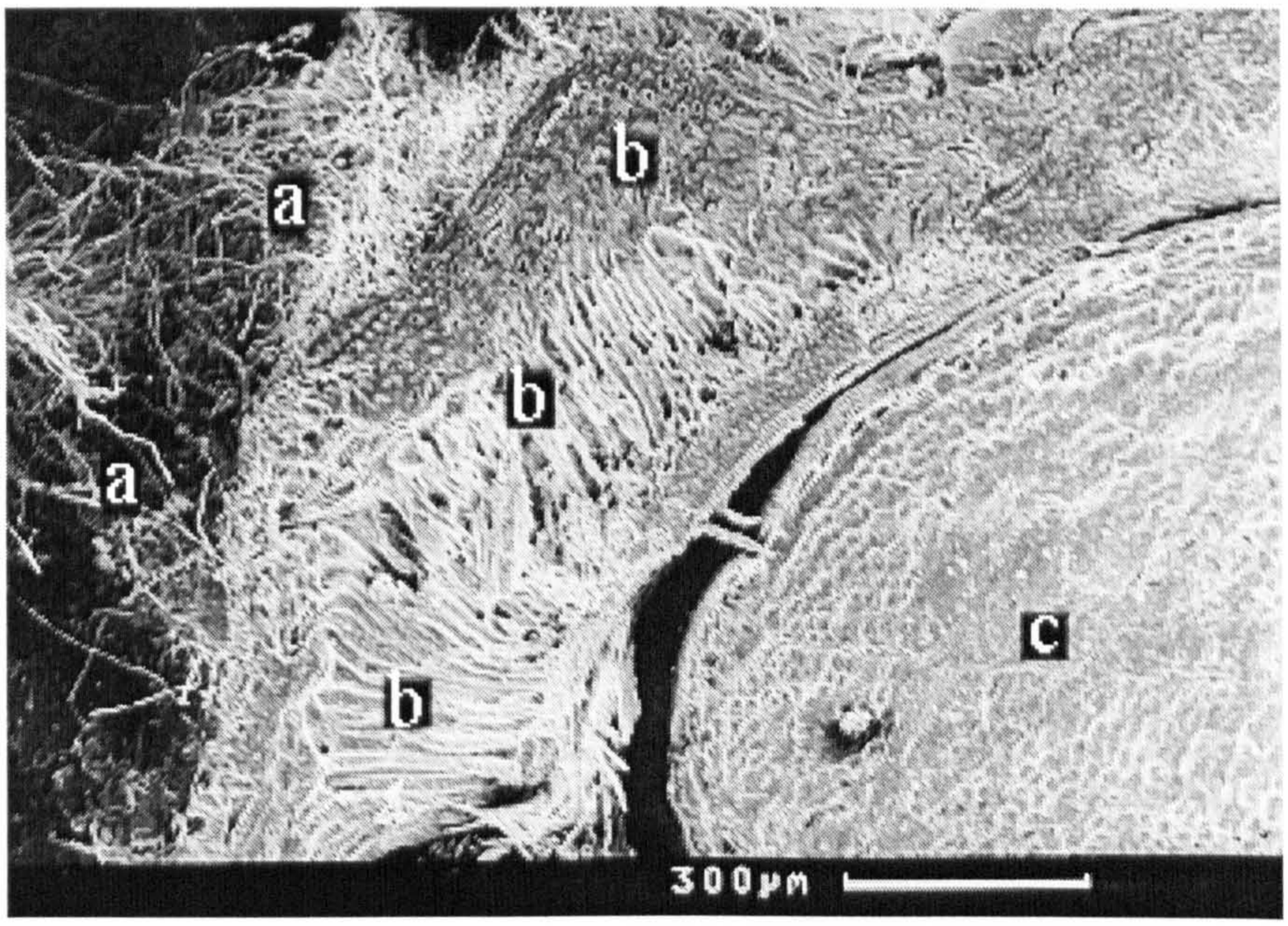


Plate 4.18 Incubation of a *Rosa corymbifera* 'Laxa' seed under identical conditions to that in plate 4.16.

However this was cut after incubation under moist conditions. It shows that the microbial structures seen in plate 4.16 orginate and grow on the outer surface of the seed coat (a). They do not penetrate through to the embryo (c) or the seed coat (b).

Plates 4.7 - 4.12 show that seed placed into the pretreatment mix at setup has a very low microbial coverage on the seed coat surface. This correseponds to the results in section 2.2 where it was shown that it was *Rosa corymbifera* 'Laxa' seed which carried the microbial loading into the pretreatment mix. Evidence of microbes on seeds was also found in the anatomy studies (section 3.3).

Whilst the microbial loading is small, the potential for growth is great. Plates 4.13 - 4.18 show that when the seed was placed into conditions conducive for microbial growth, the seed coat became highly covered with fungi after only 48 hours.

4.4.2.2 Seed sampled 3 weeks into pretreatment

The commercially pretreated samples are shown in plates 4.19 to 4.26

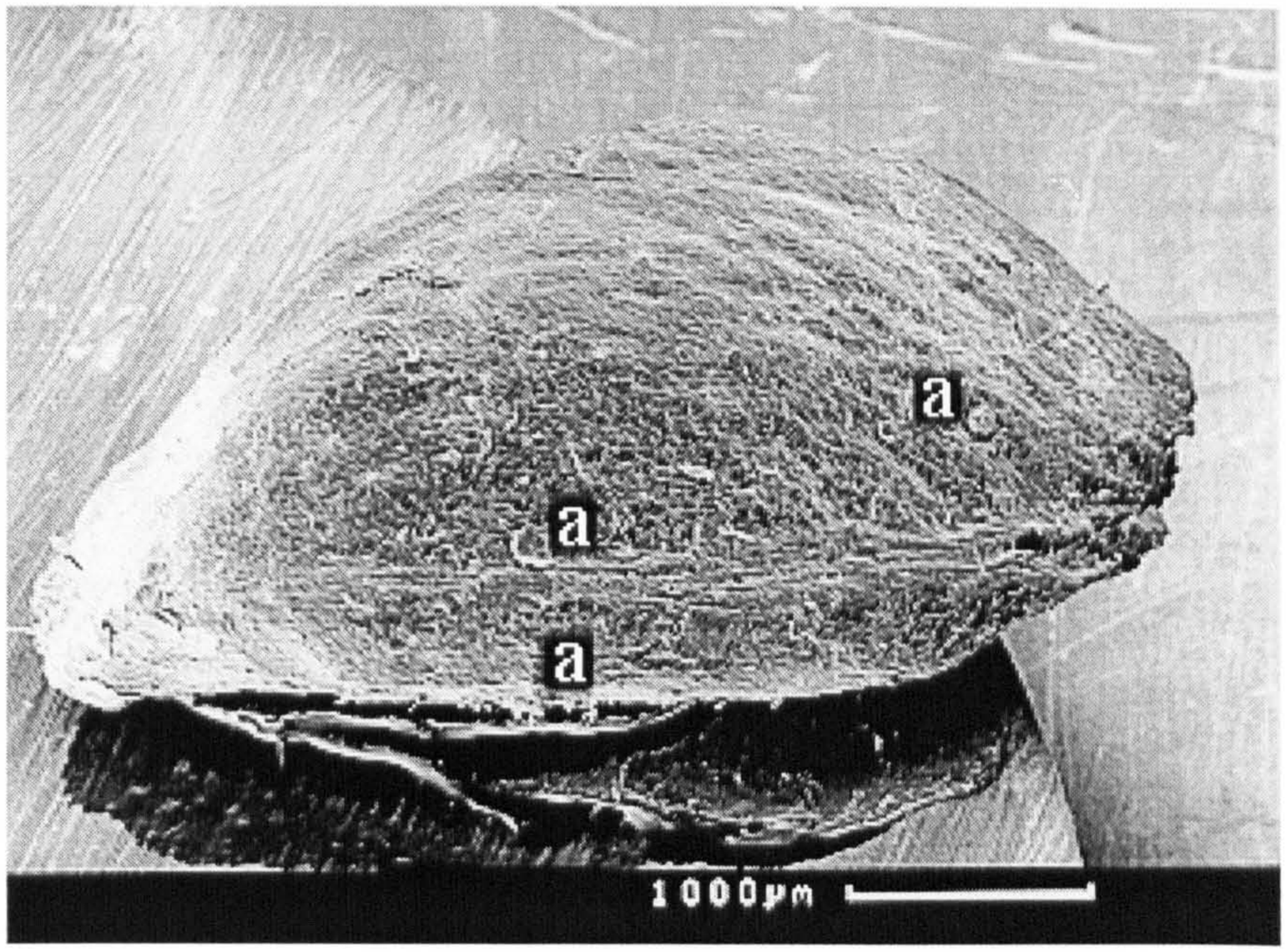


Plate 4.19 This seed of *Rosa corymbifera* 'Laxa' appears relatively free from microbes. however closer inspection reveals pockets of microbial growth across the seed (a).

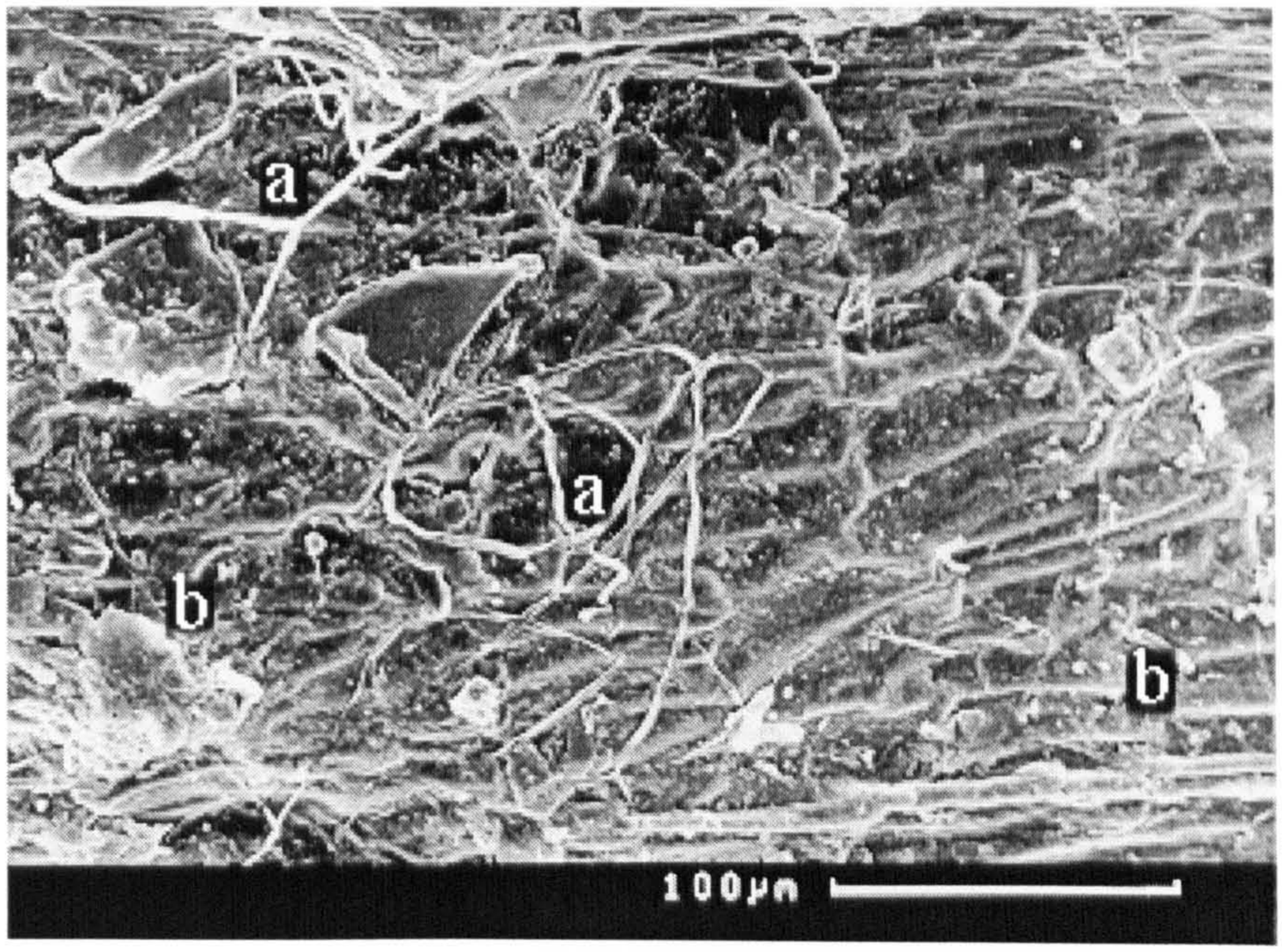


Plate 4.20 One such area of microbial activity magnified from plate 4.19. Fungal hyphae (a) and spores (b) can be seen across the surface.

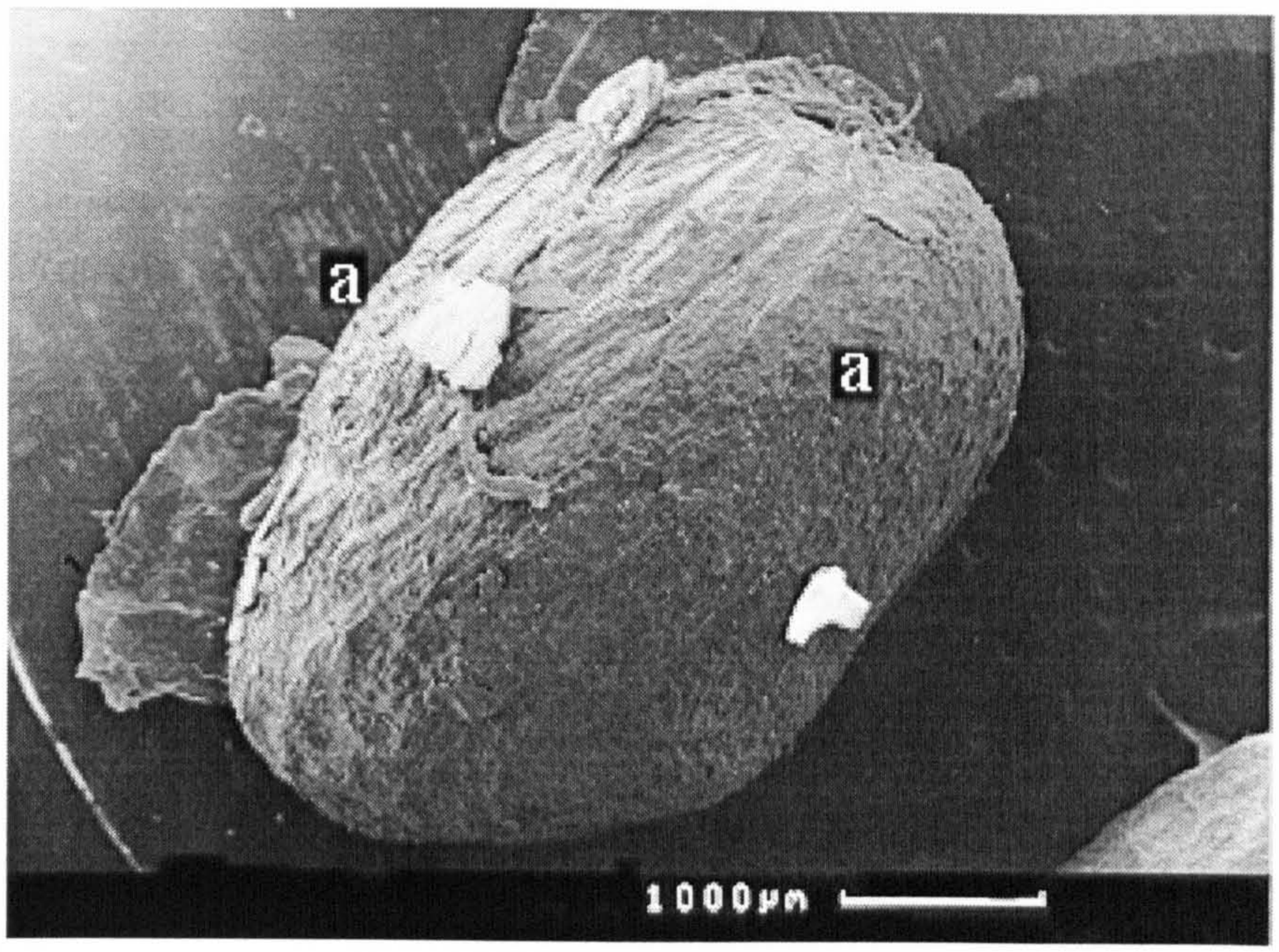


Plate 4.21 The seed in this plate has little microbial growth on the seed coat. However, microbes are present, albeit at a very low level (a).

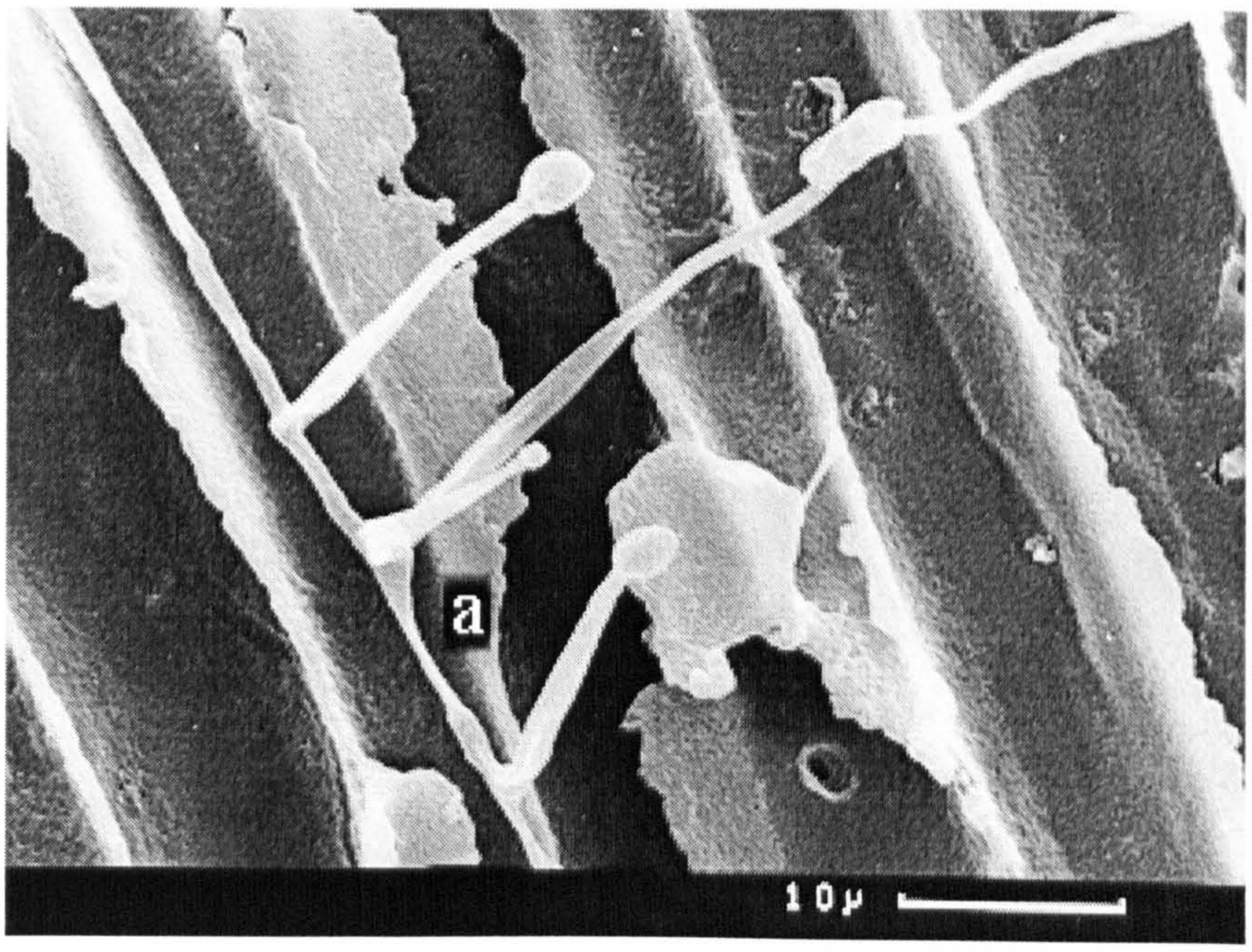


Plate 4.22 Careful examination of the seed coat in plate 4.21 revealed fungal hyphae (a), as shown in this plate.

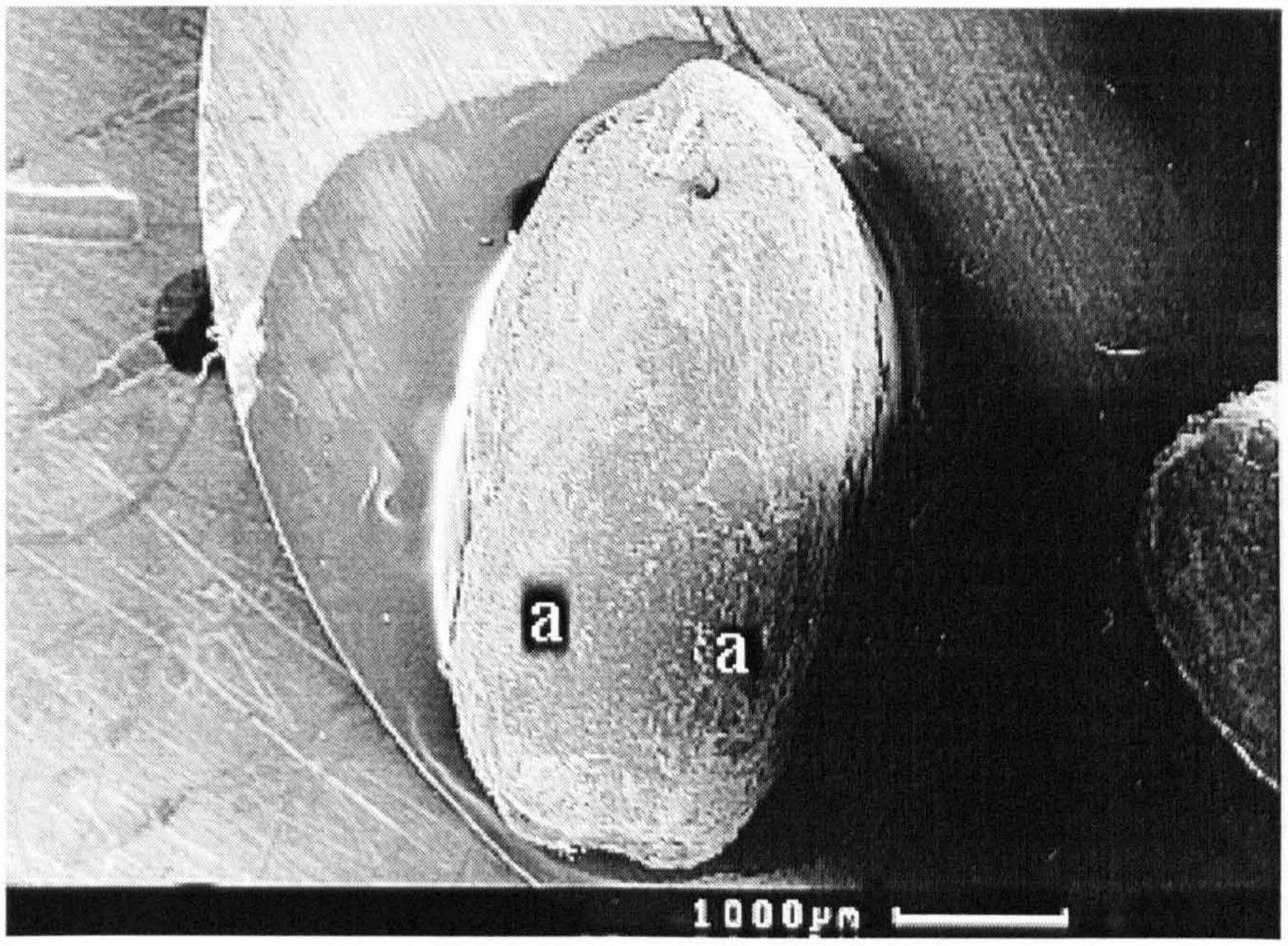


Plate 4.23 This seed also appears clean of microbial growth, although one or two areas of microbial growth are visible (a).

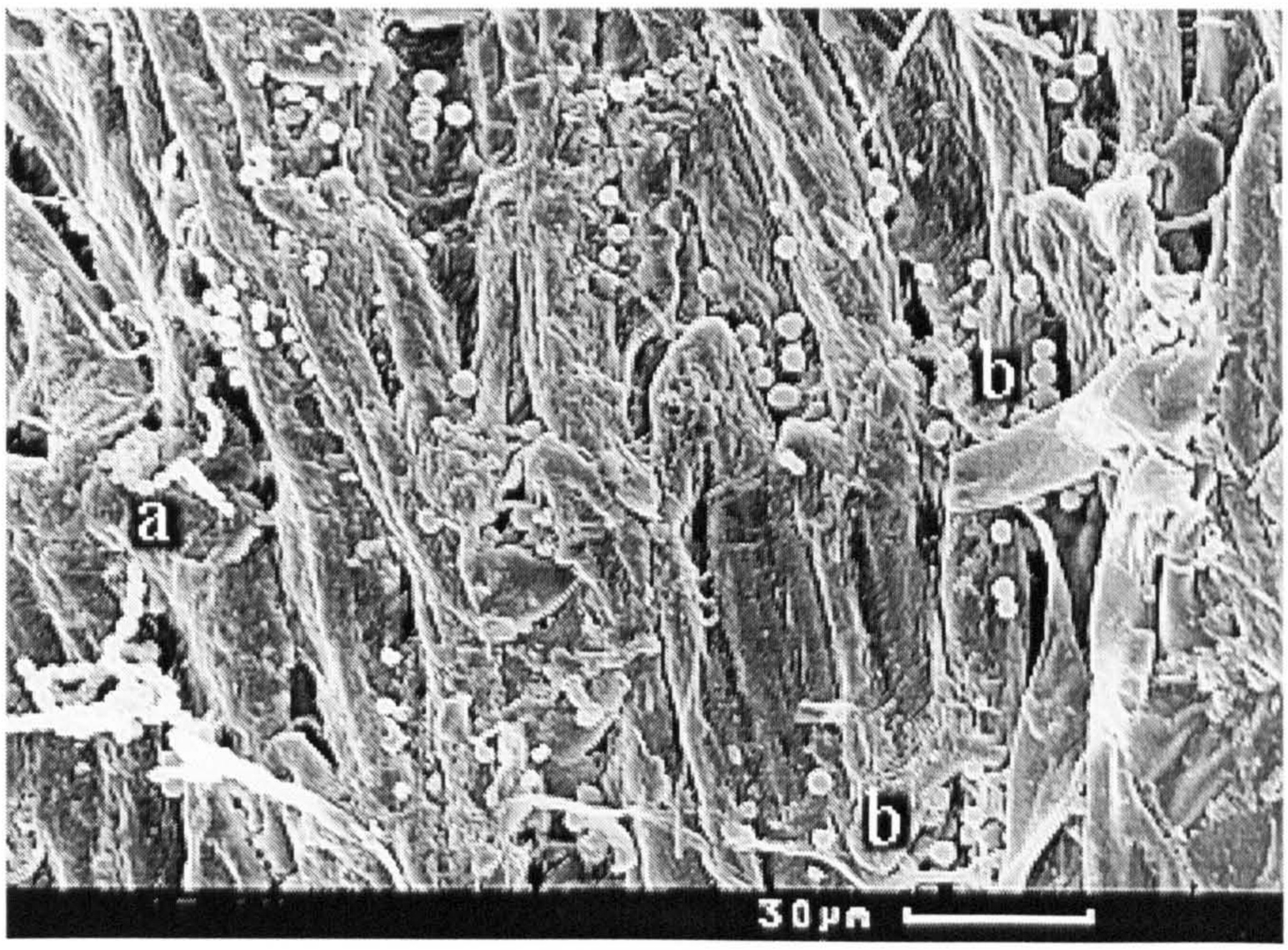


Plate 4.24 Closer examination of the seed in plate 4.23.
This shows some evidence of microbial loading. Chains of spores (a) and single spores (b) are visible.

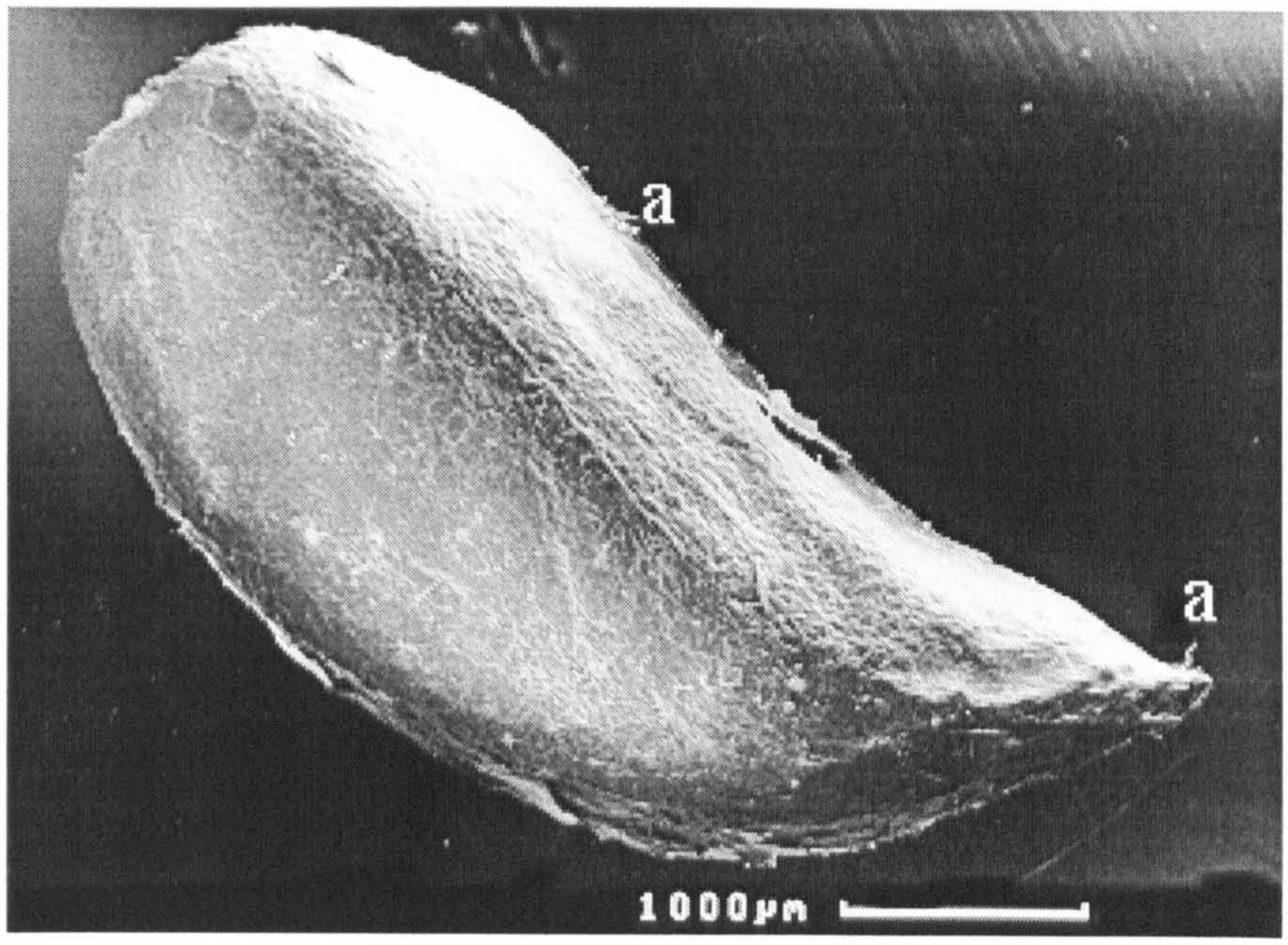


Plate 4.25 As with the other commercially pretreated seeds, there are a few signs of microbial loading on this seed coat.
Some are visible on the top edge of the seed (a).

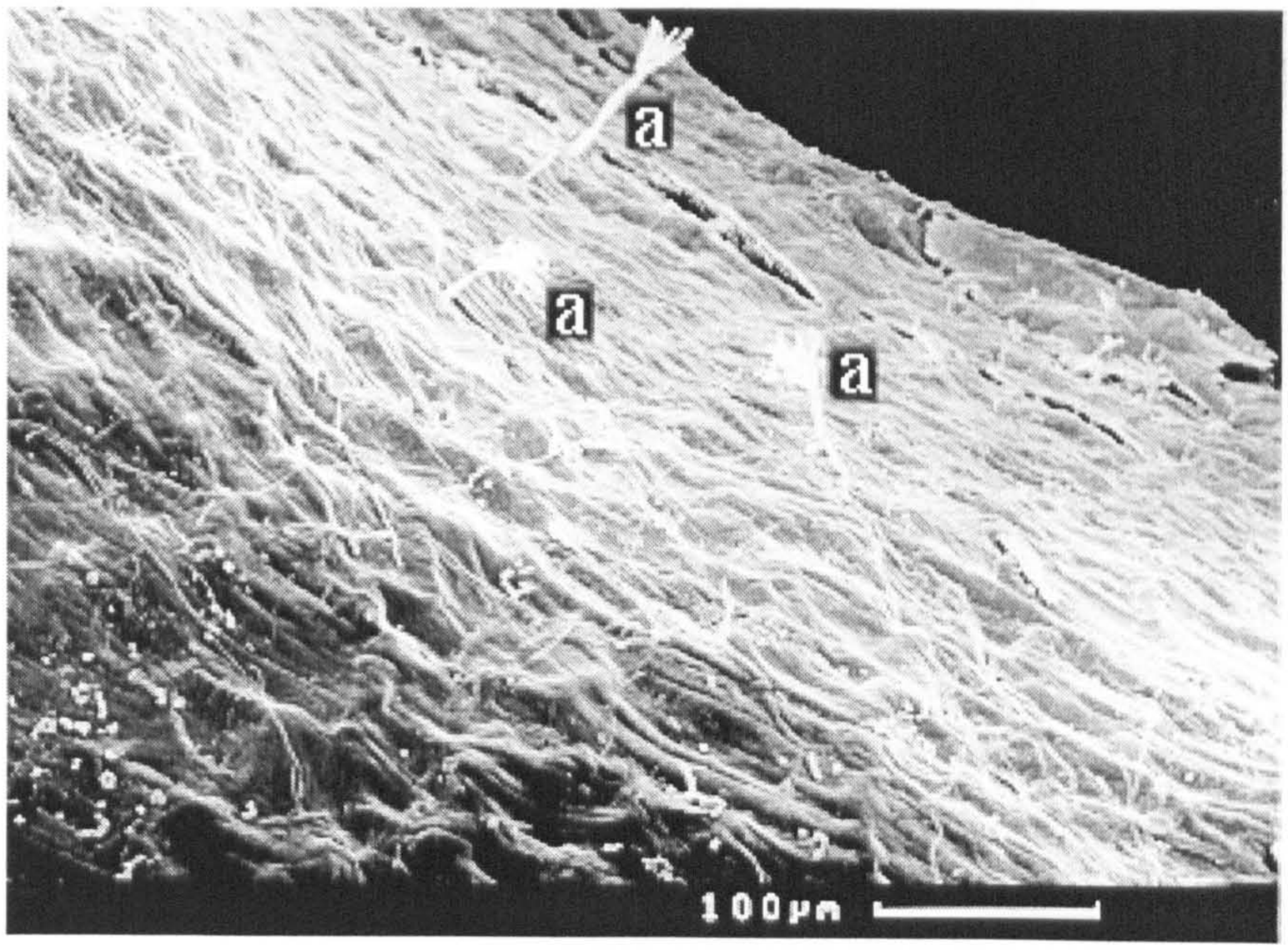


Plate 4.26 The top edge of the seed in plate 4.25 is magnified here.
The fruiting bodies of a *Penicillium* species are clearly visible (a).

The Garotta pretreated samples from week 3 are shown in plates 4.27 to 4.41

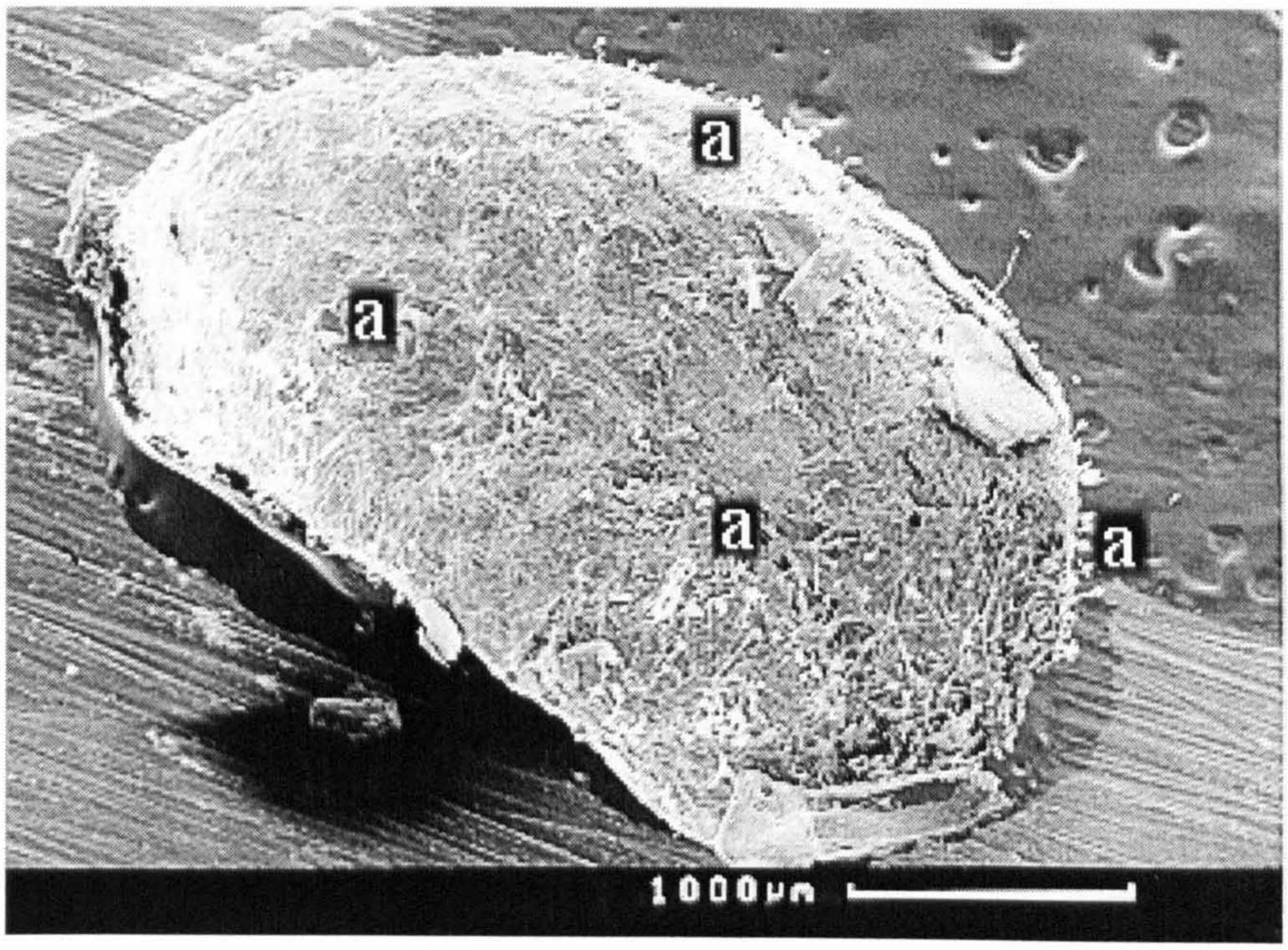


Plate 4.27 This Garotta pretreated *Rosa corymbifera* 'Laxa' seed is covered with fungal colonies (a).
The entire surface is covered with *Penicillium* species which contrasts with the equivalent commercially pretreated seed which had very little microbial growth.

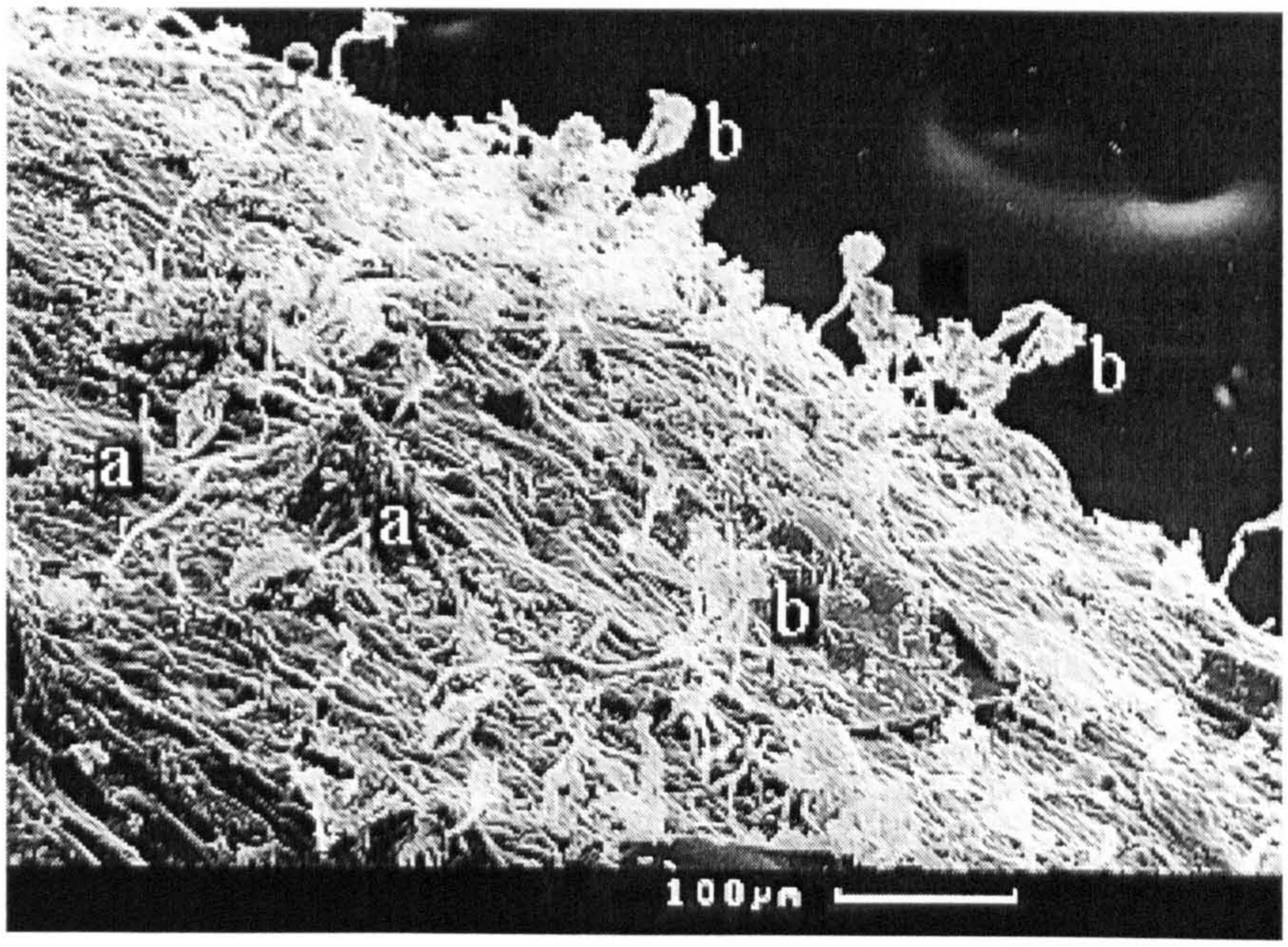


Plate 4.28 The fungal colonies in plate 4.27 are shown magnified here.
The fungal growth is shown very clearly, and the hyphae (a) and fruiting bodies (b) of a *Penicillium* species are easily defined.

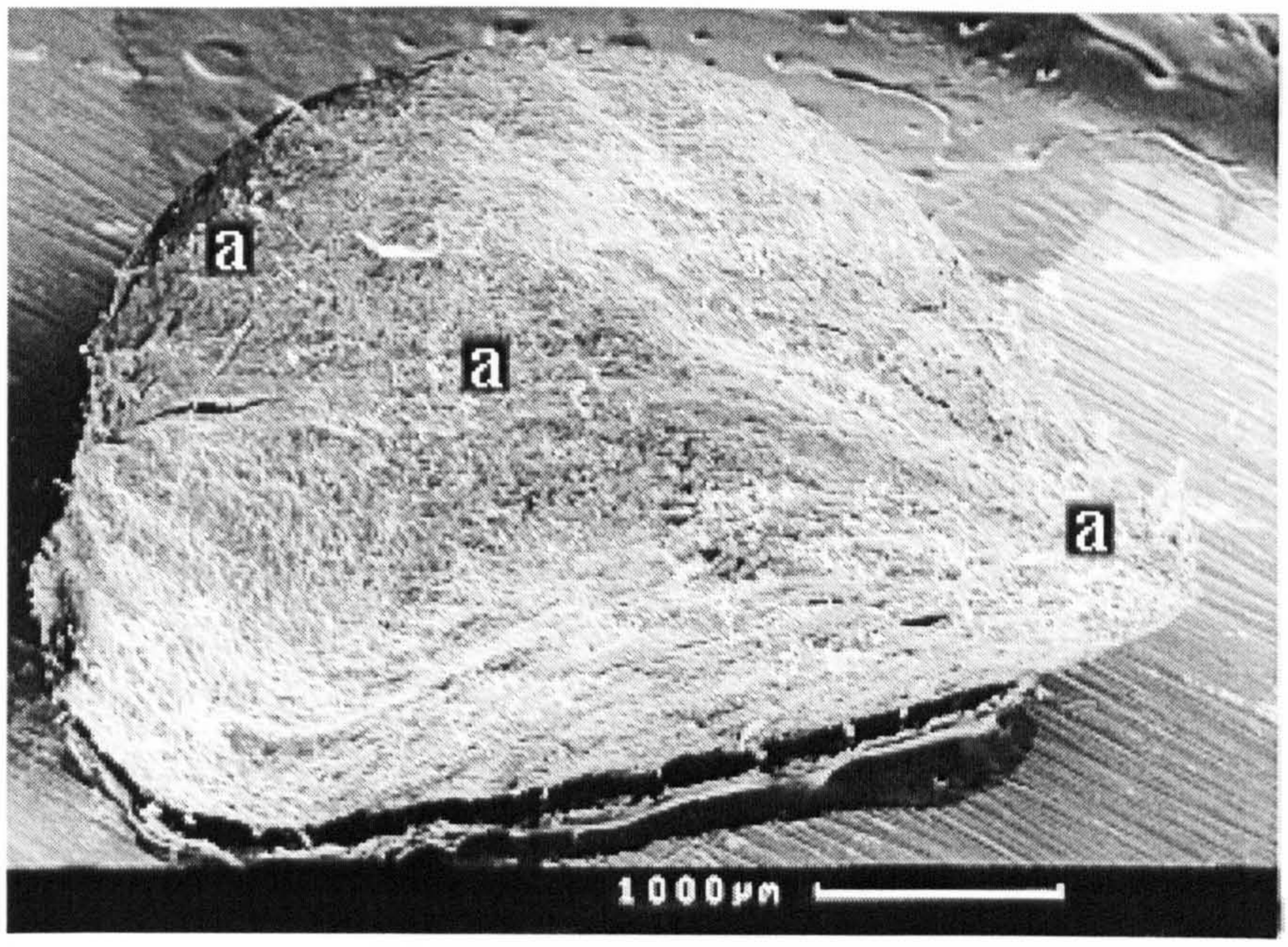


Plate 4.29 The whole surface of the seed coat is covered with fungal colonies (a). The very dense areas are particularly visible (a).

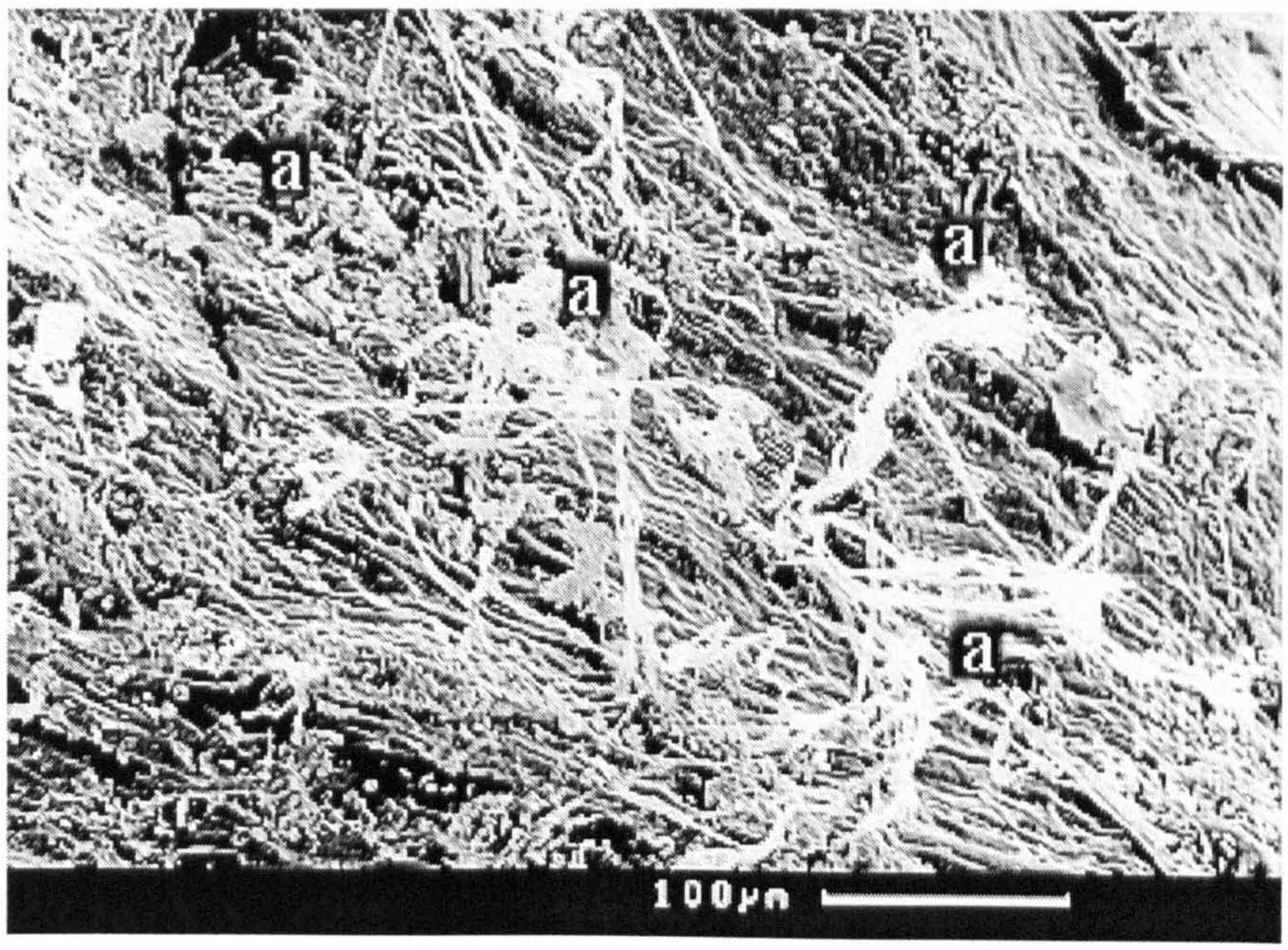


Plate 4.30 Closer inspection of the *Rosa corymbifera* 'Laxa' seed in plate 4.29 reveals the extent of the fungal growth.
Hyphae can be seen across the seed coat surface (a).

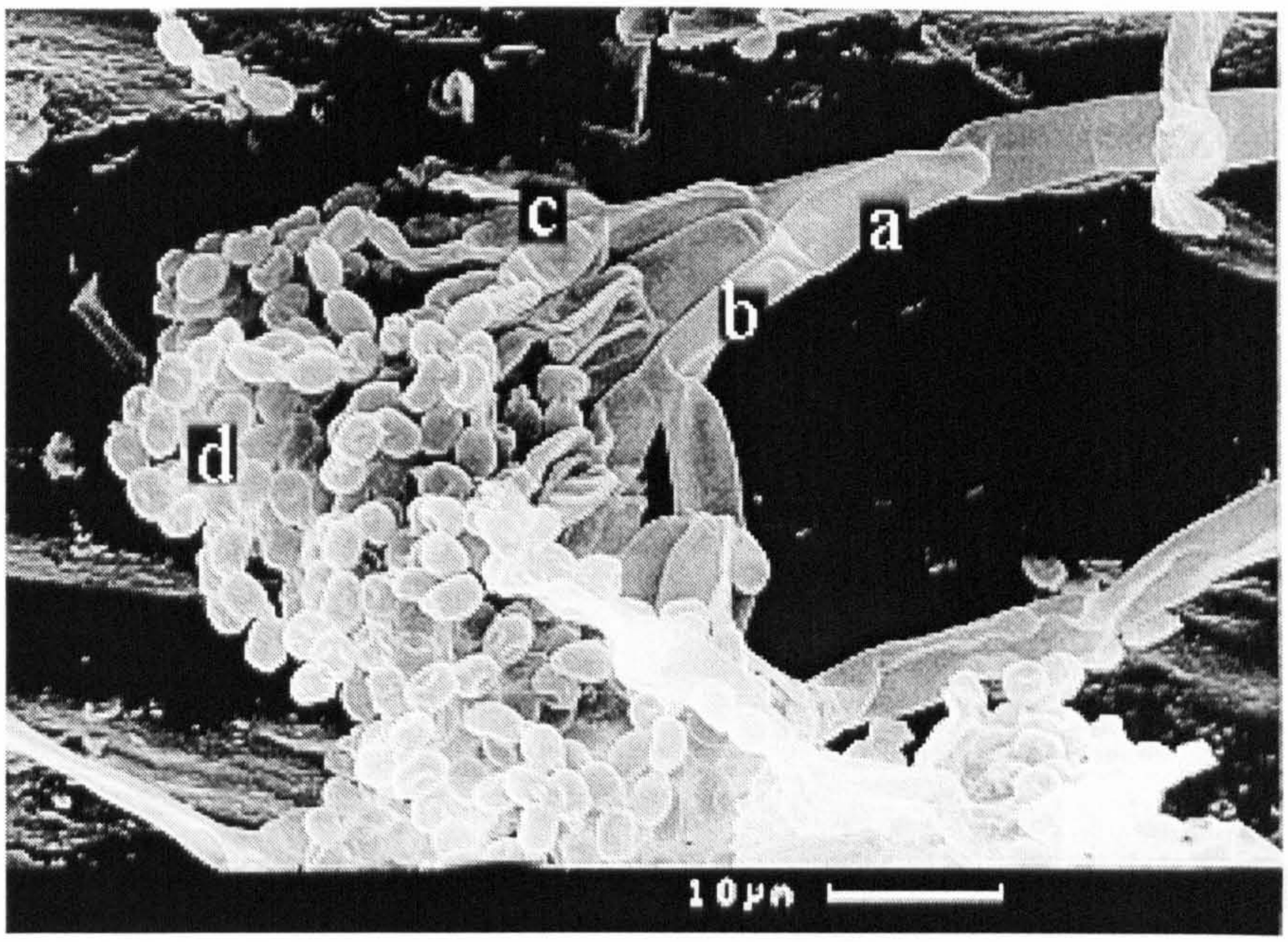


Plate 4.31 The detail of the *Penicllium* species seen covering the seed coat in plates 4.29 and 4.30 is shown here.
The fine structure of the fruiting form of this species is very clear. The structures of the ramus (a), metula (b), phialide (c) and spores (d) are very distinctive.

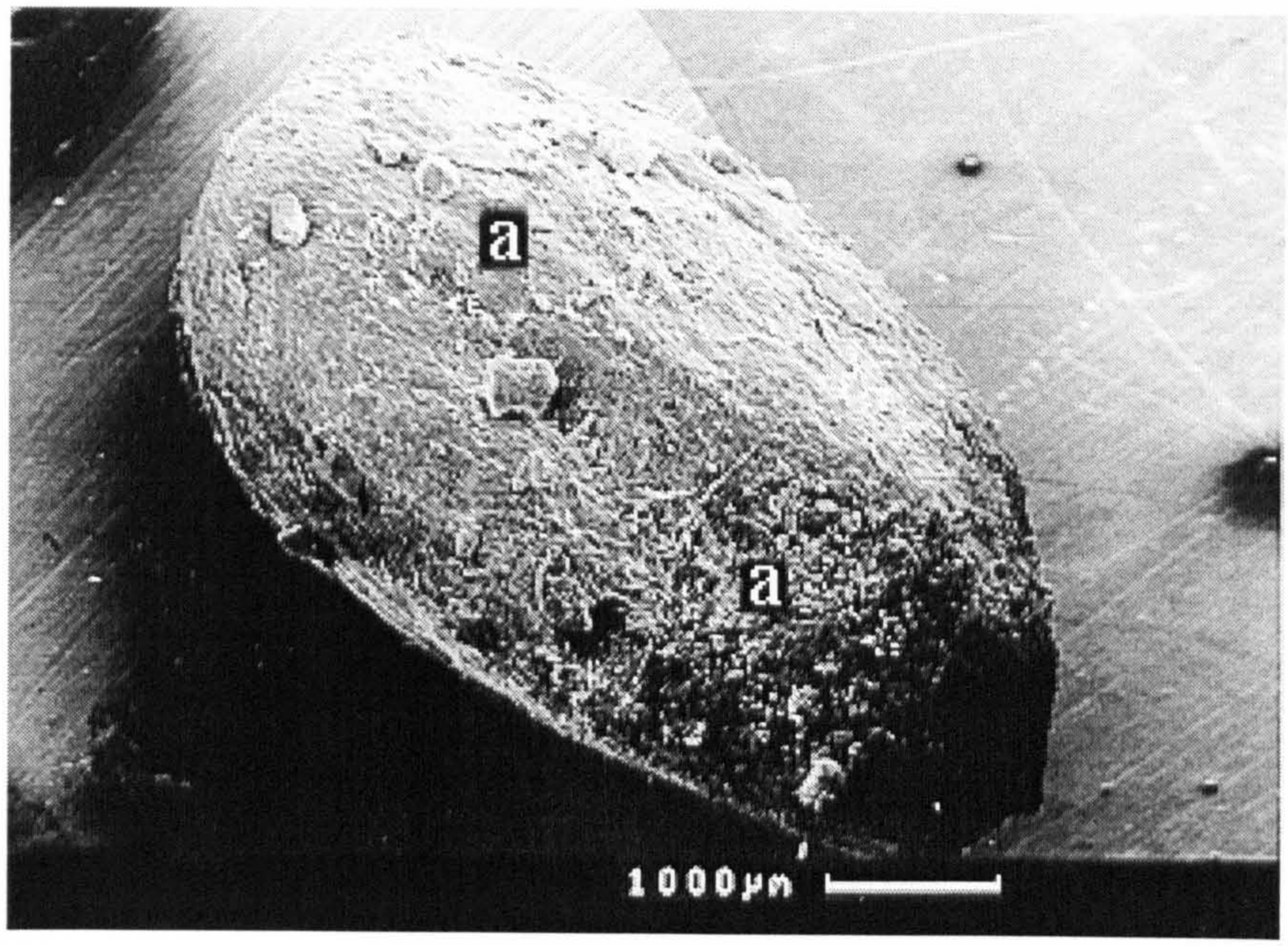


Plate 4.32 This seed of *Rosa corymbifera* 'Laxa' is also covered with fungal growth (a).

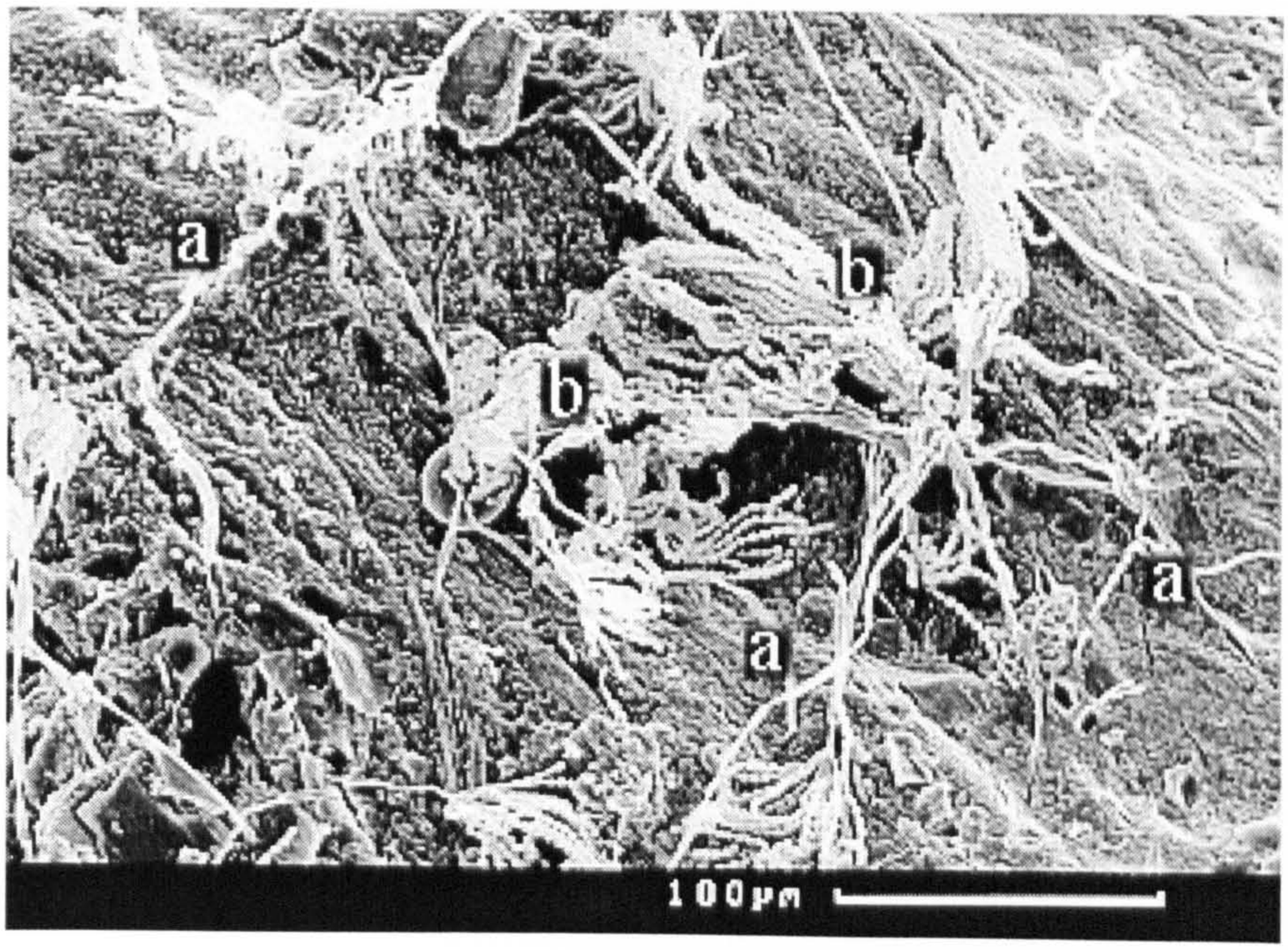


Plate 4.33 The hyphae (a) and fruiting bodies (b) of a *Penicillium* species are very easily discernible when they are magnified from the seed in plate 4.32.

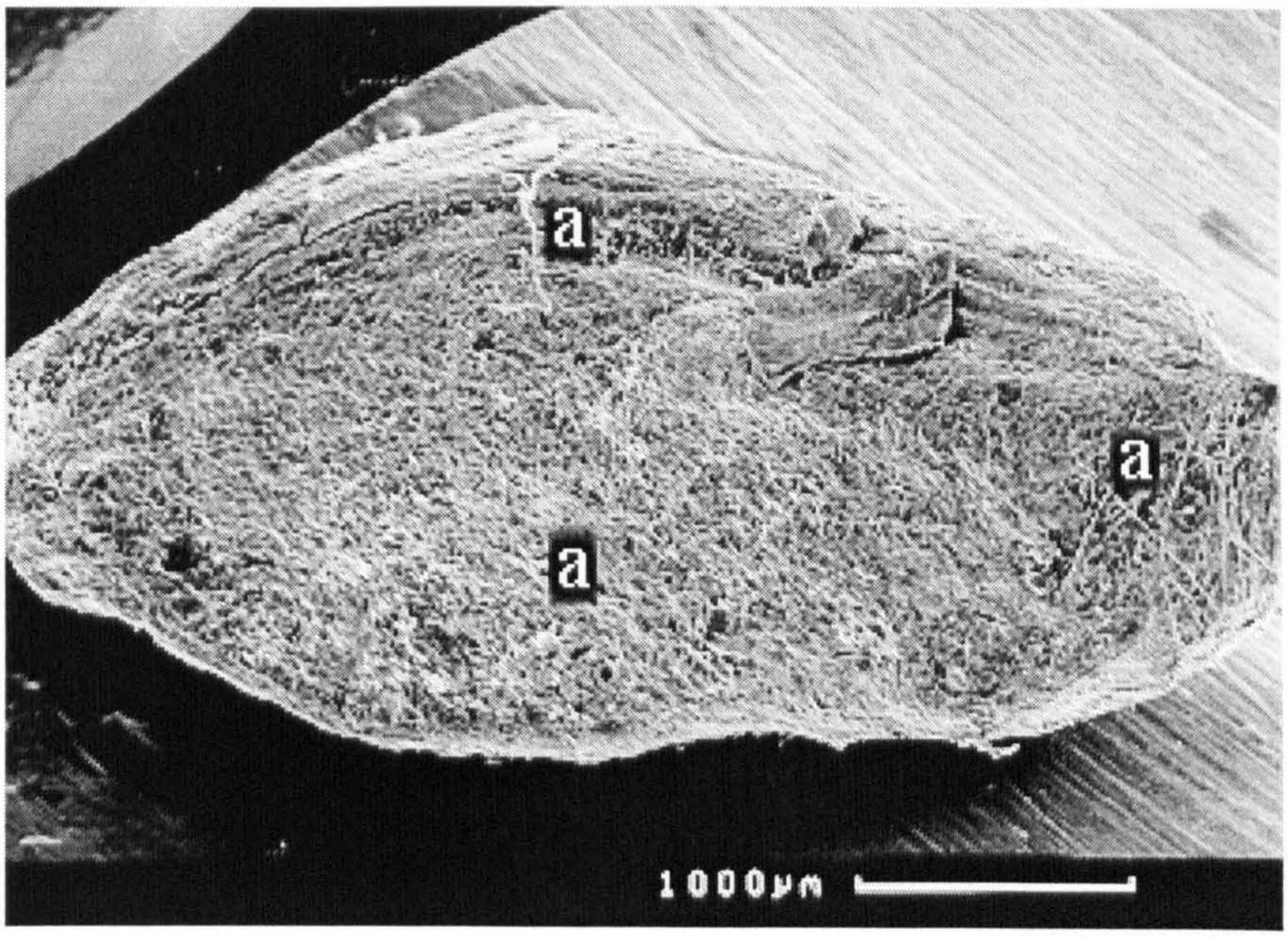


Plate 4.34 This seed of *Rosa corymbifera* 'Laxa', which was also pretreated with Garotta, is also covered with microbial growth (a).

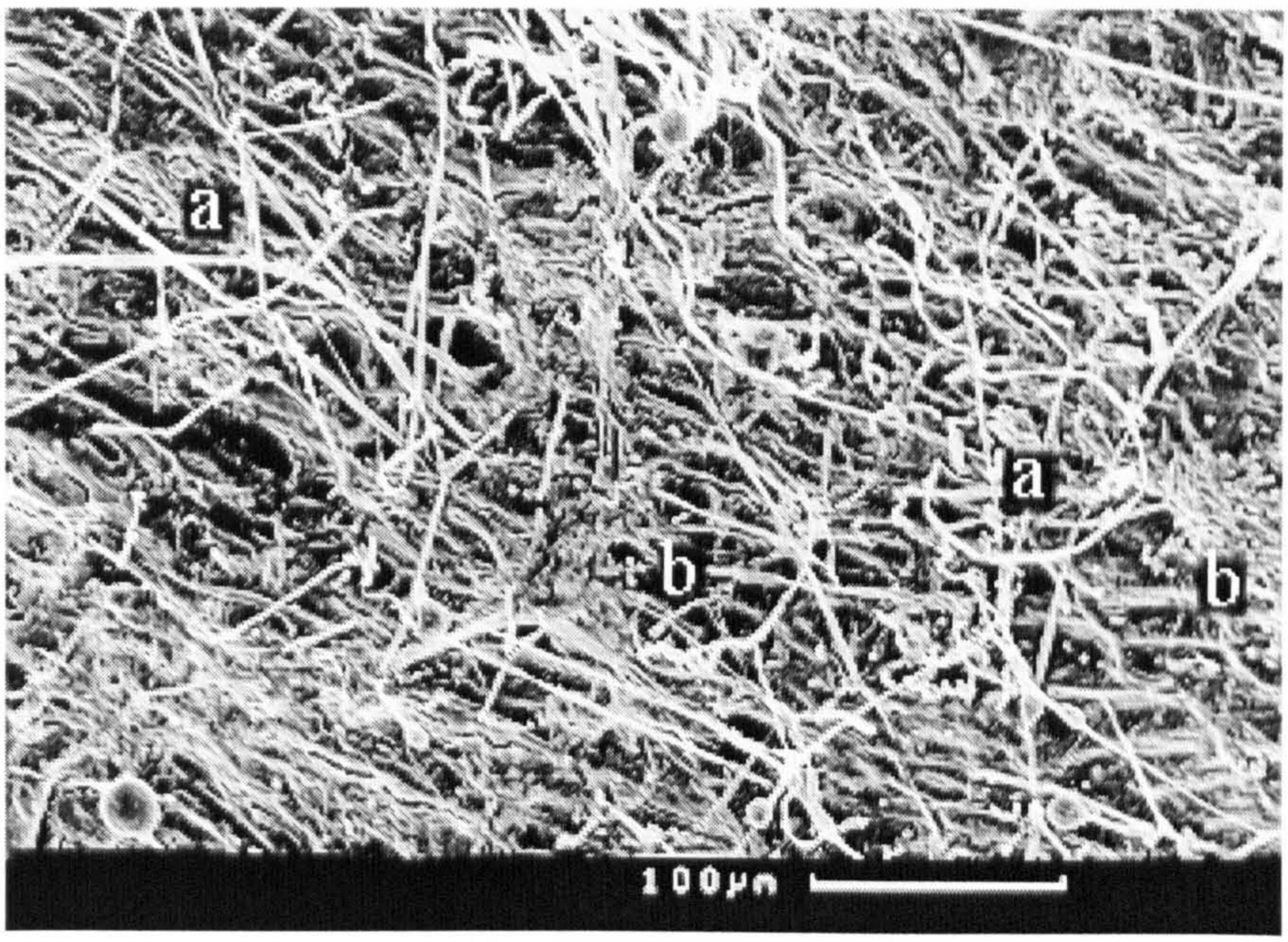


Plate 4.35 Enlarging one area of the seed from the previous plate reveals dense hyphal growth (a) and many spores (b).

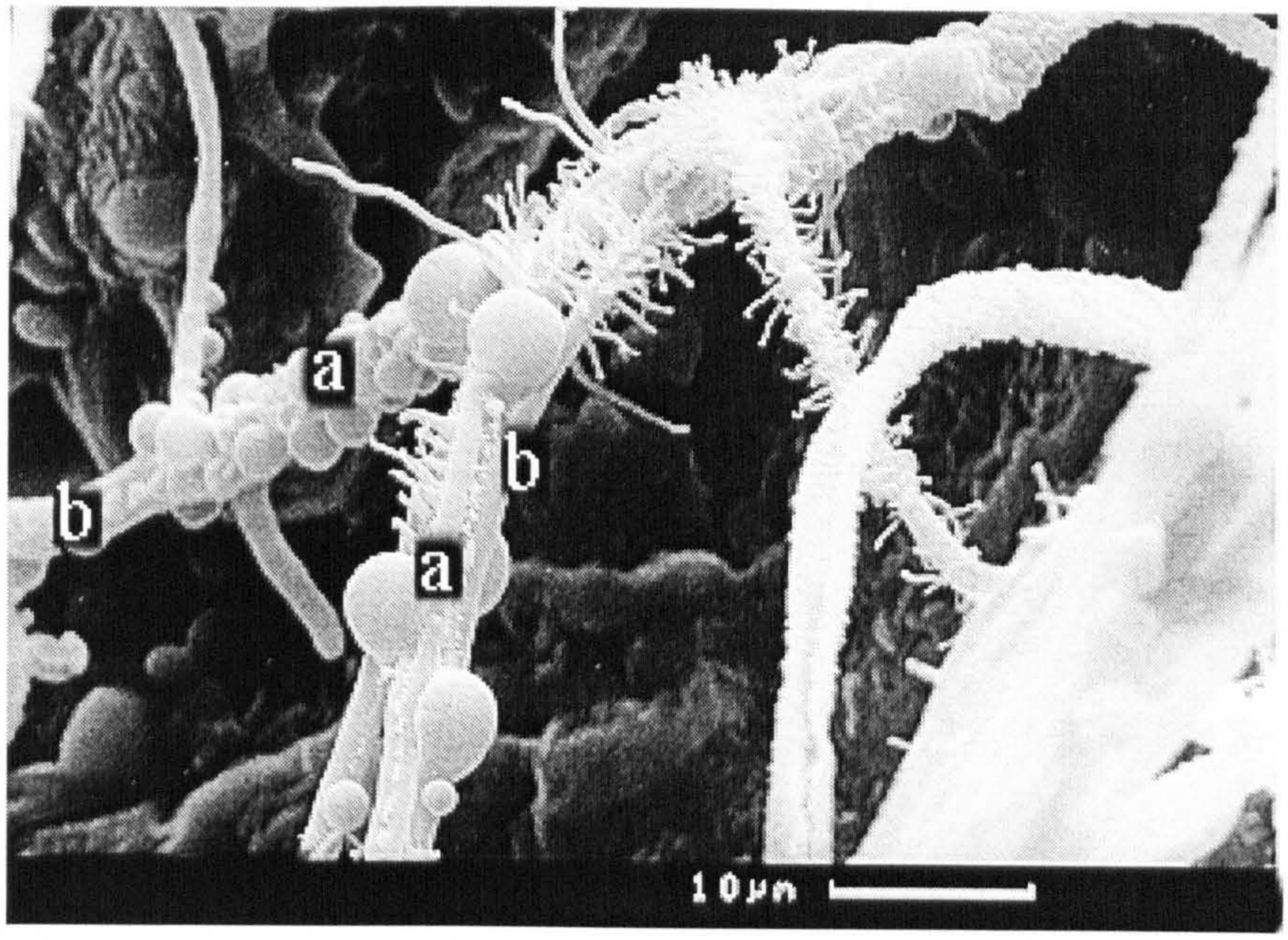


Plate 4.36 In this plate the structures in plate 4.35 are shown enlarged. The cylindrical structures are new spores (a) forming from the existing hyphae (b).

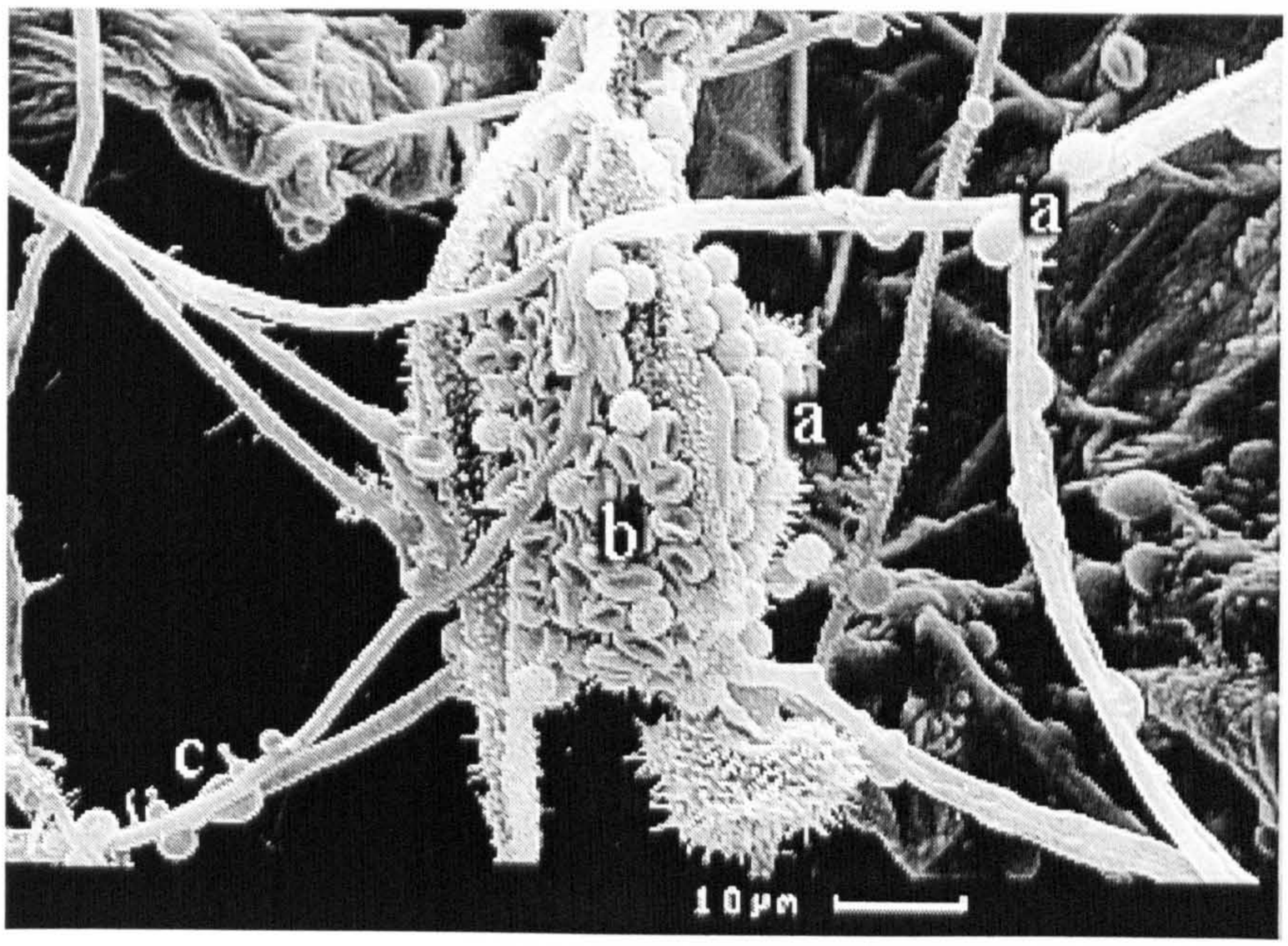


Plate 4.37 Another part of the same seed as in plate 4.36 shows another fungal structure. Again many spores have formed (a), some of which have collapsed (b) due to the drying of the preparation technique. In the bottom left hand corner there are also yeast-like buds forming (c).

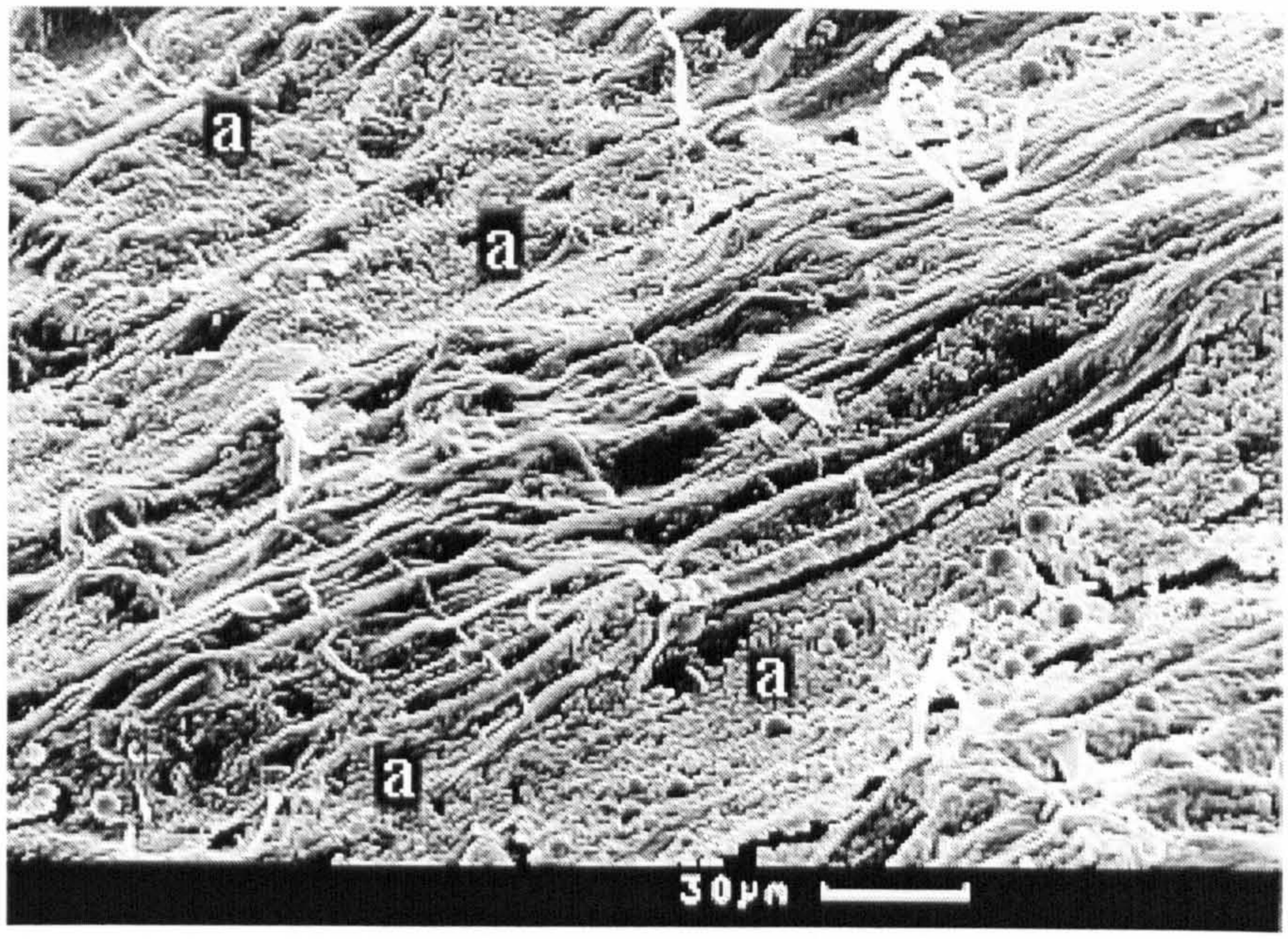


Plate 4.38 This plate (and plate 4.39) were taken from another Garotta pretreated seed. Hundreds of spores of varying sizes are visible (a). They appear like sheets of spores or yeast cells closely associated with the seed coat.

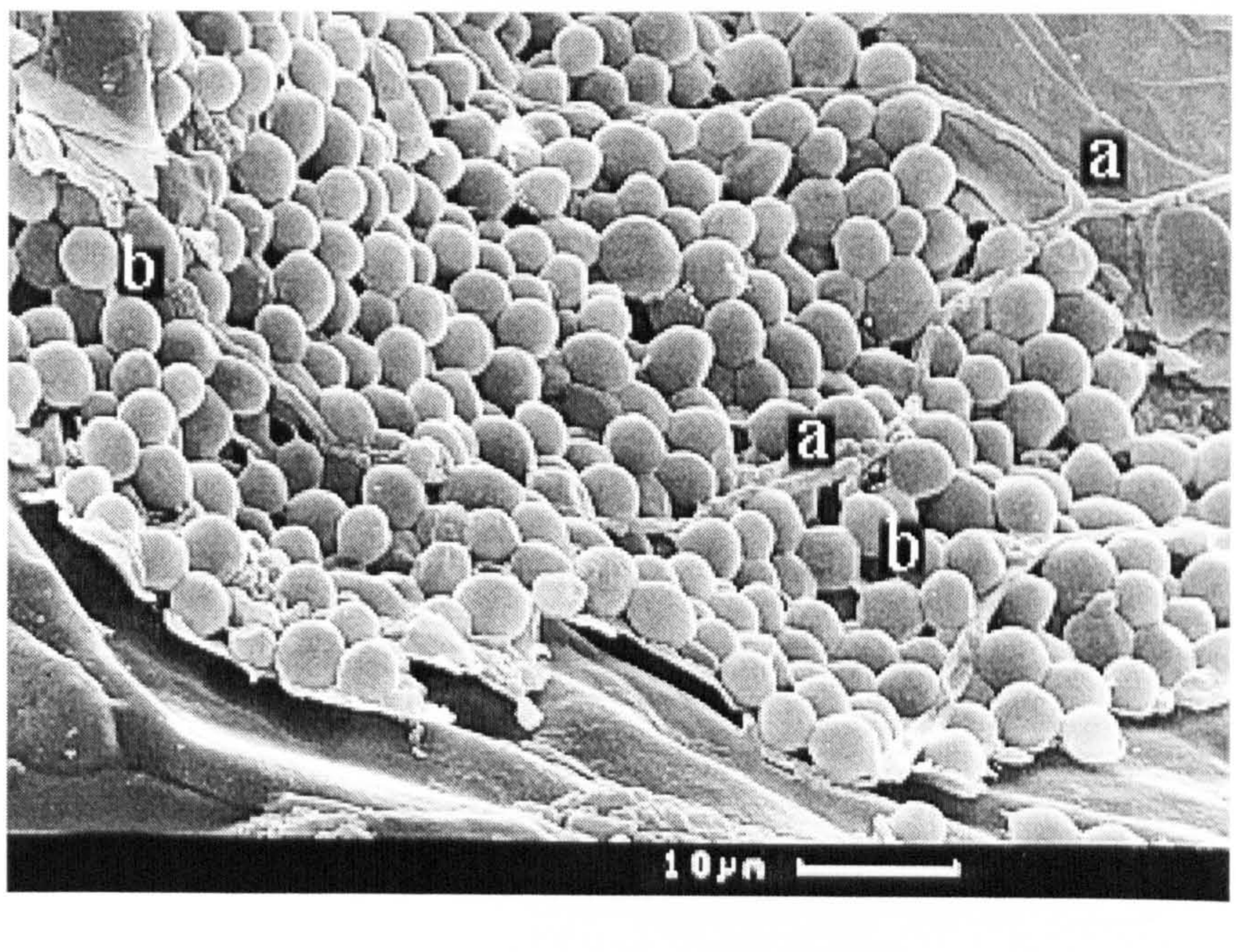


Plate 4.39 Looking closer at these 'sheets', it can be seen that hyphae (a) are running through them.
Some of the spores or yeast cells also appear joined together (b).

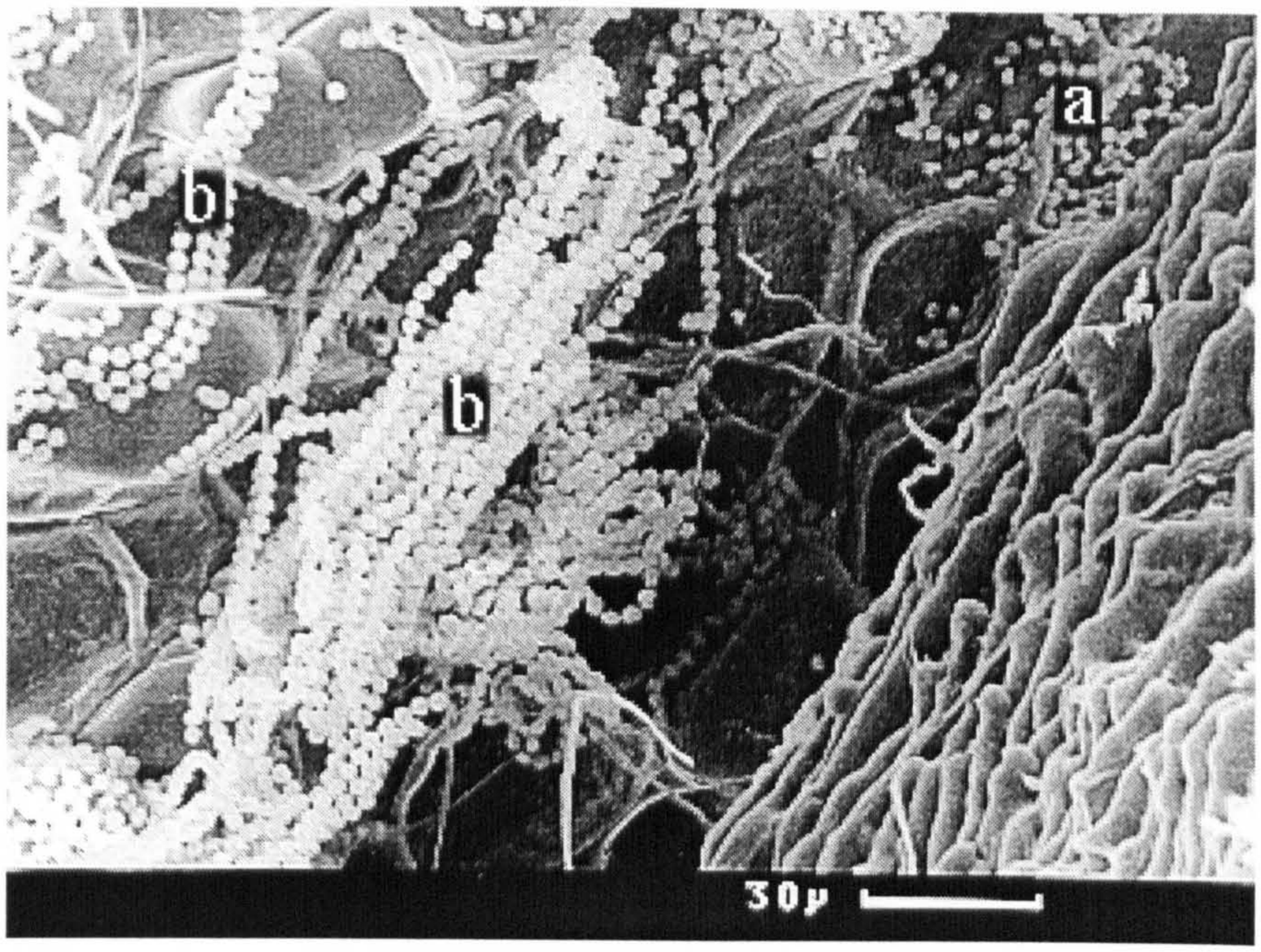


Plate 4.40 This plate was taken from another Garotta pretreated seed.
There are many single spores (a) lying on the seed coat surface, but there are also many chains of spores (b) similar to those found in many fungal species. These are likely to be from an *Aspergillus* species.

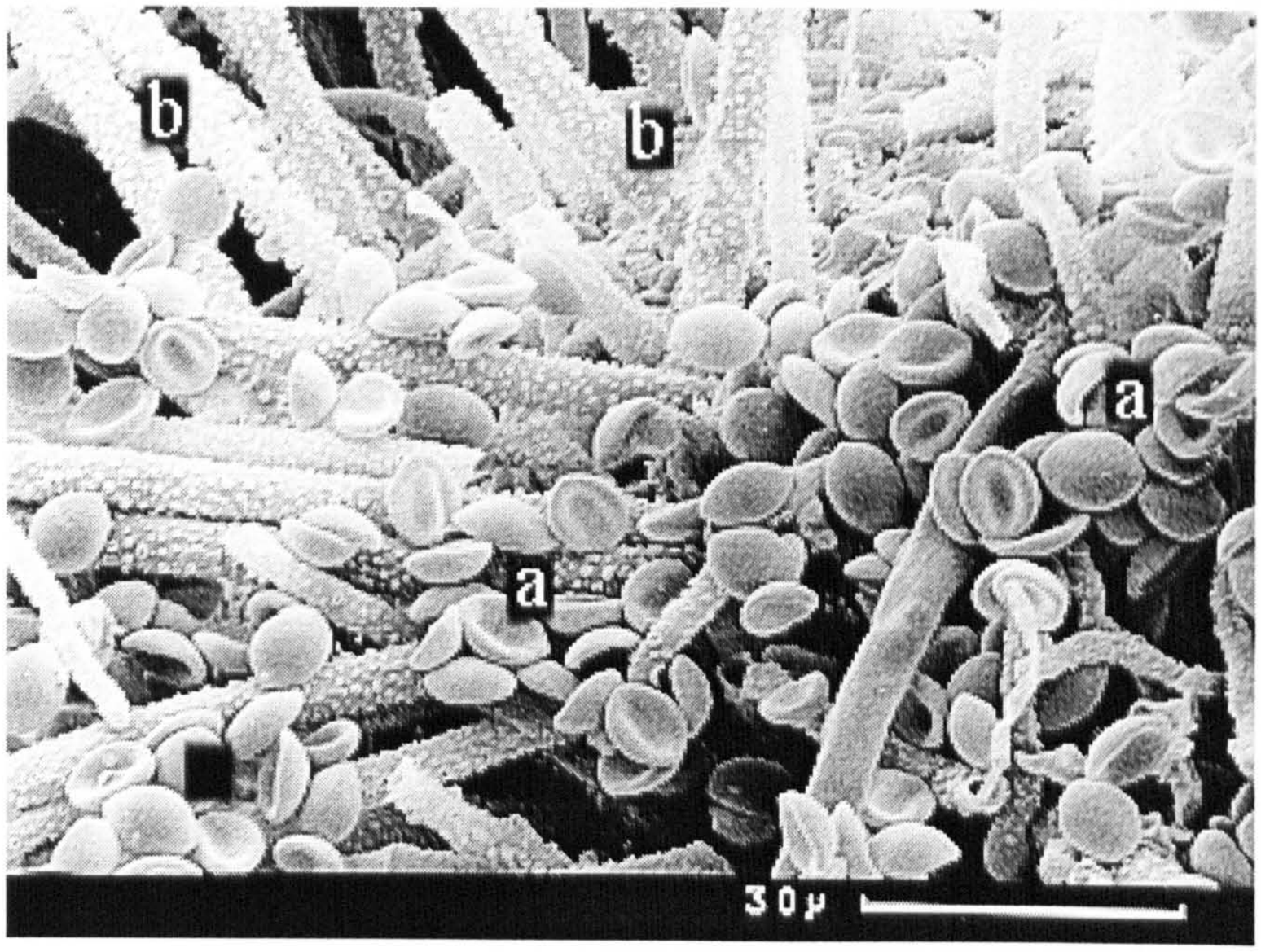


Plate 4.41 These structures were also found on a Garotta pretreated seed.
The spores are mainly collapsed (a) due to the preparation technique used on the samples. Also of interest is the appearance of the hyphae (b), being very pitted from where spores would have formed and then dispersed.

4.4.2.3 Seed sampled at 12 weeks (end of warm period)

Commercially pretreated seed is shown in plates 4.42 to 4.47.

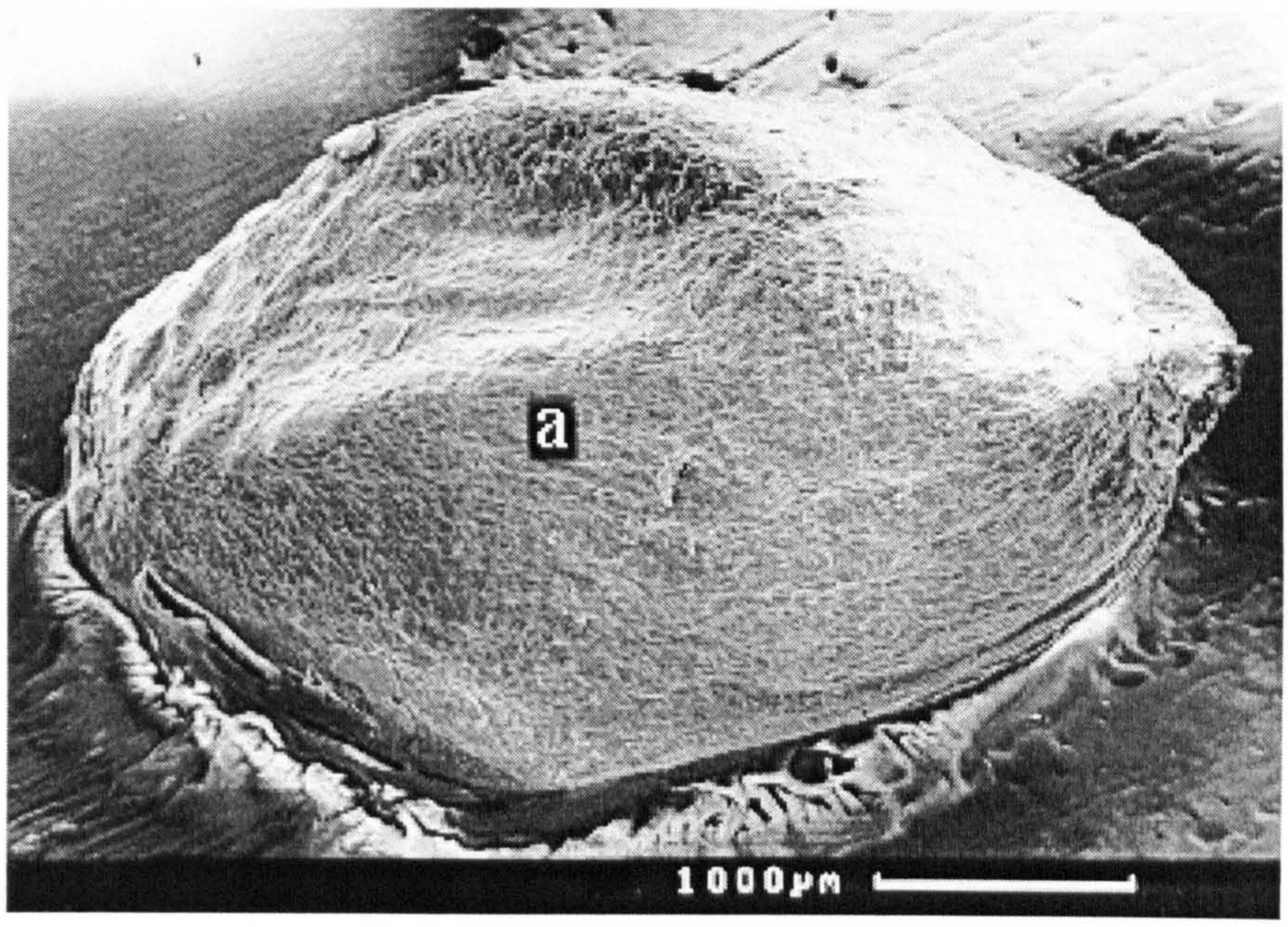


Plate 4.42 A commercially pretreated seed of *Rosa corymbifera* 'Laxa' sampled at the end of the warm period of incubation.
The seed coat is very clean with only a few patches of microbes (a).

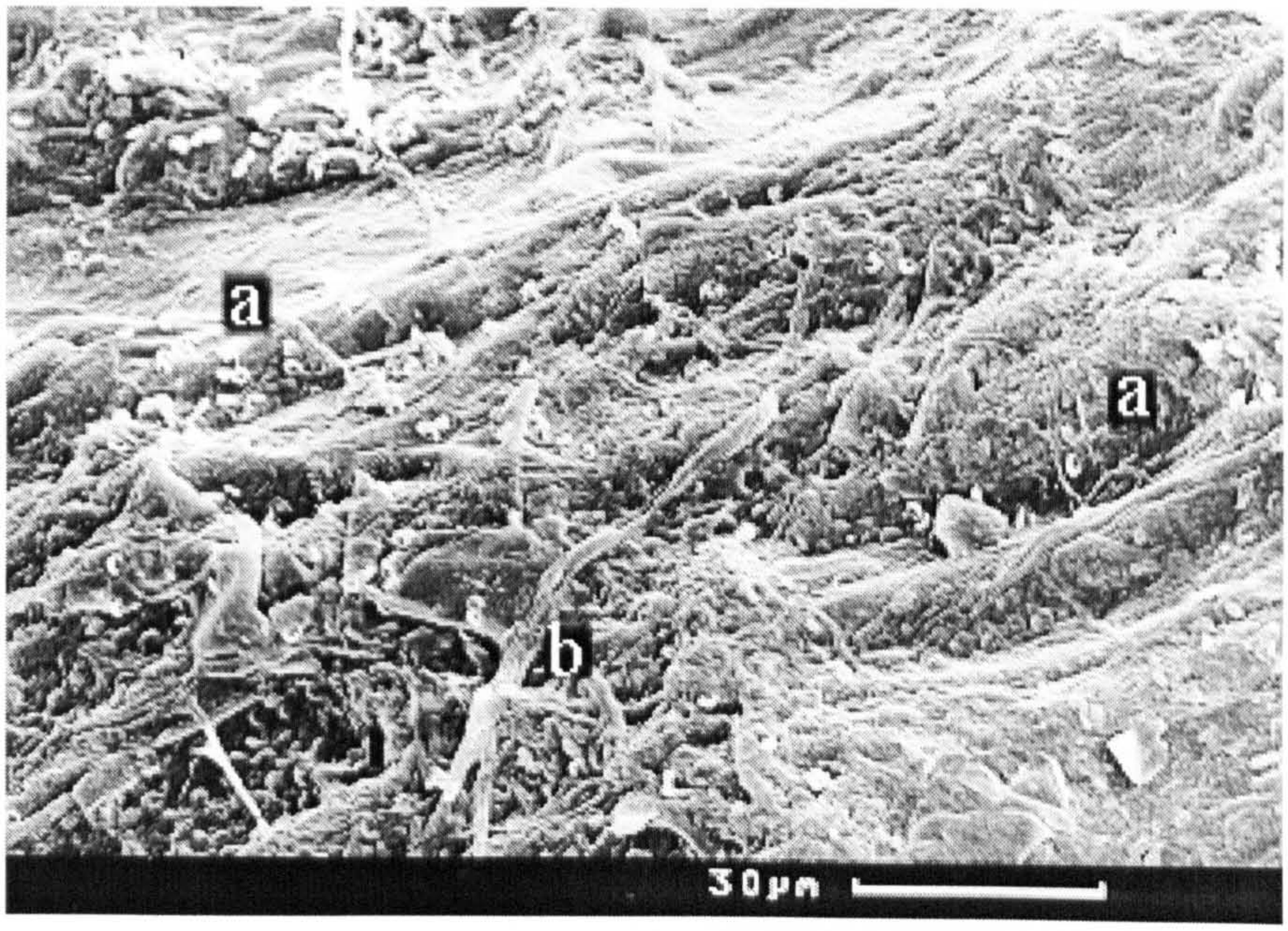


Plate 4.43 Fungal structures magnified from plate 4.42.

Spores are thinly scattered over the seed surface (a), with some hyphae present (b).

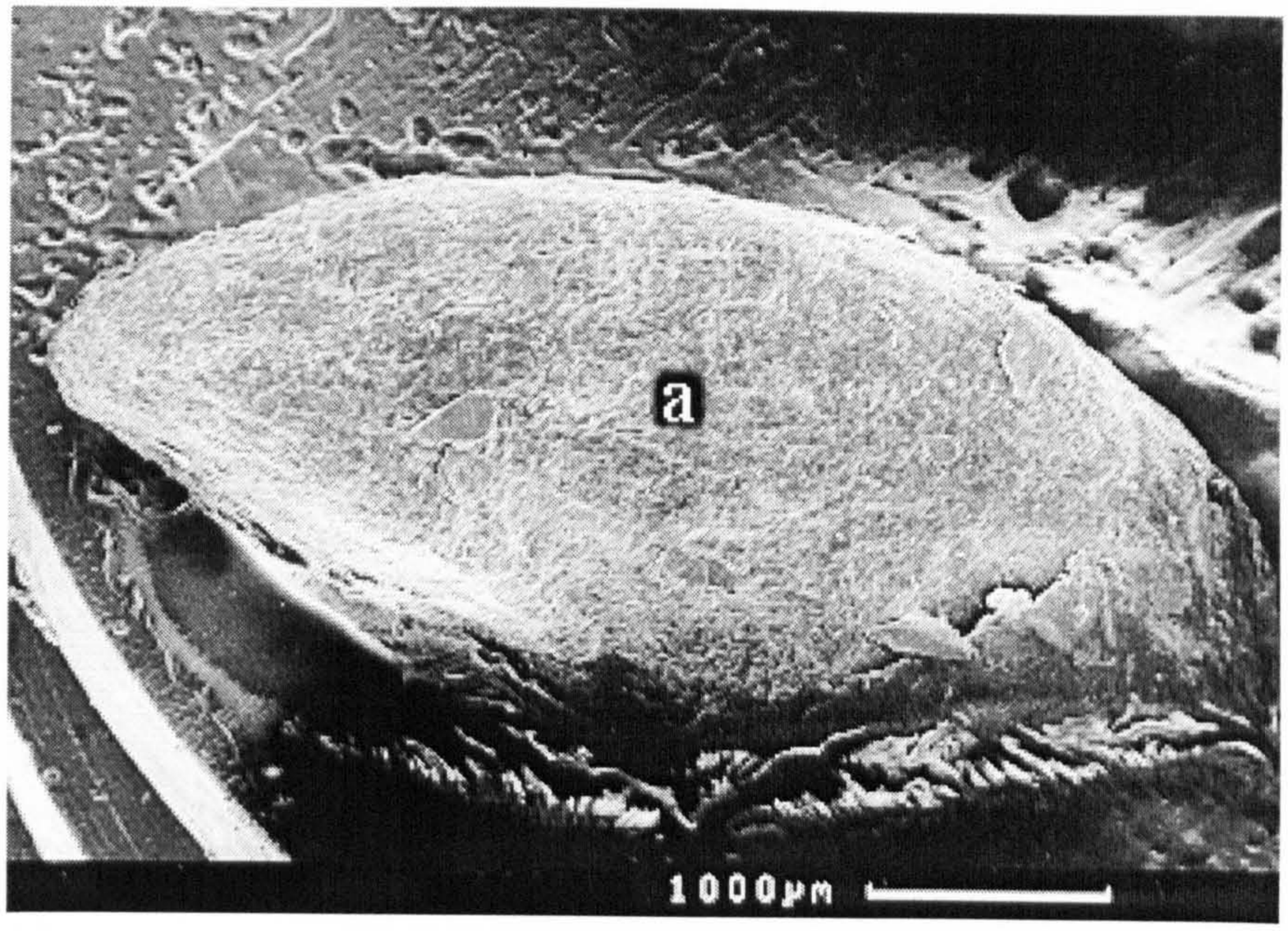


Plate 4.44 Commercially pretreated seed of *Rosa corymbifera* 'Laxa'.

This seed also has little obvious microbial activity associated with the seed coat (a).

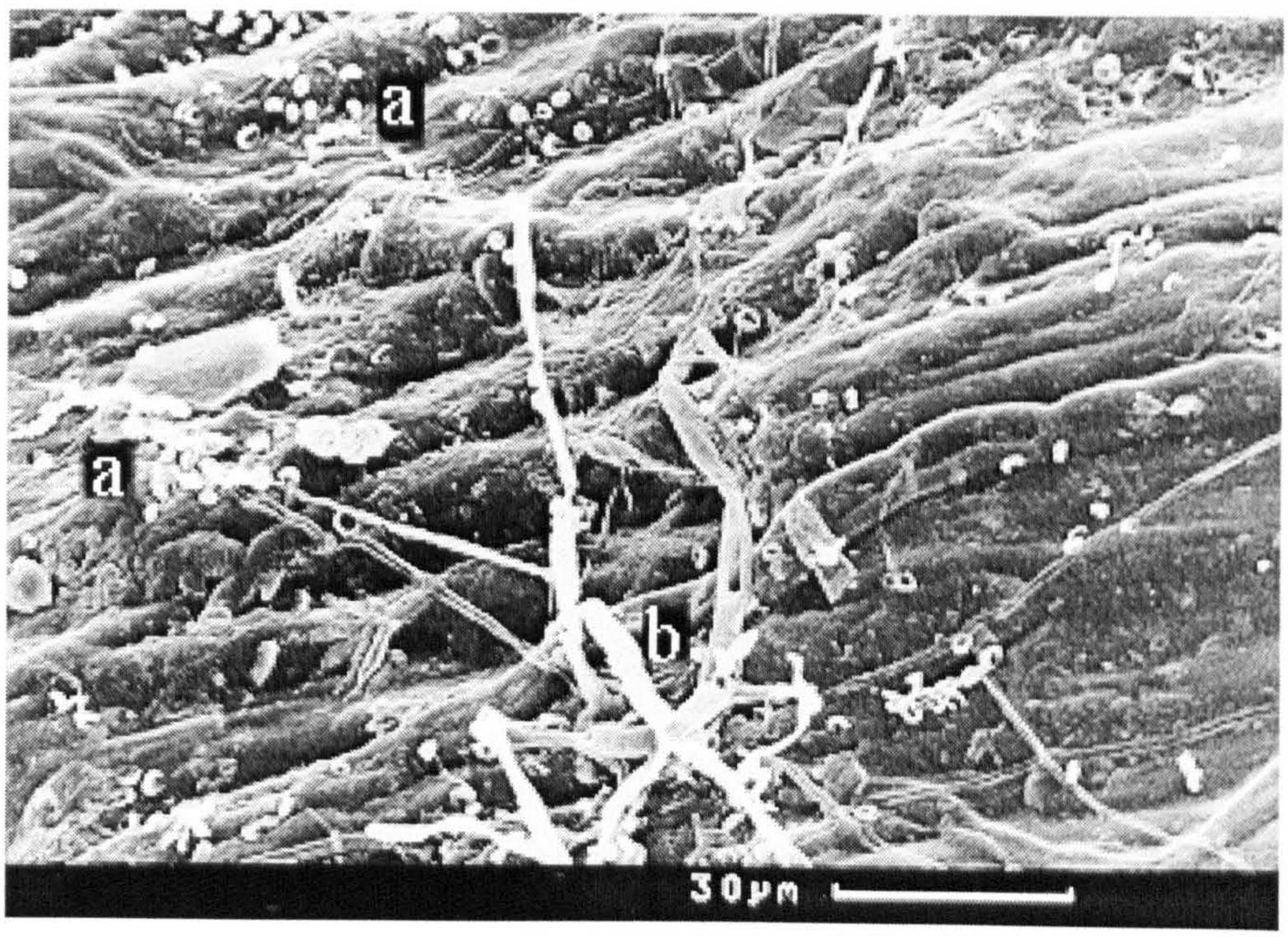


Plate 4.45 An area of plate 4.44 magnified to show microbial structures.

Fungal spores (a) and hyphae (b) are present, but in low numbers. There is little evidence of microbial growth (i.e. fresh spores and spreading hyphae).

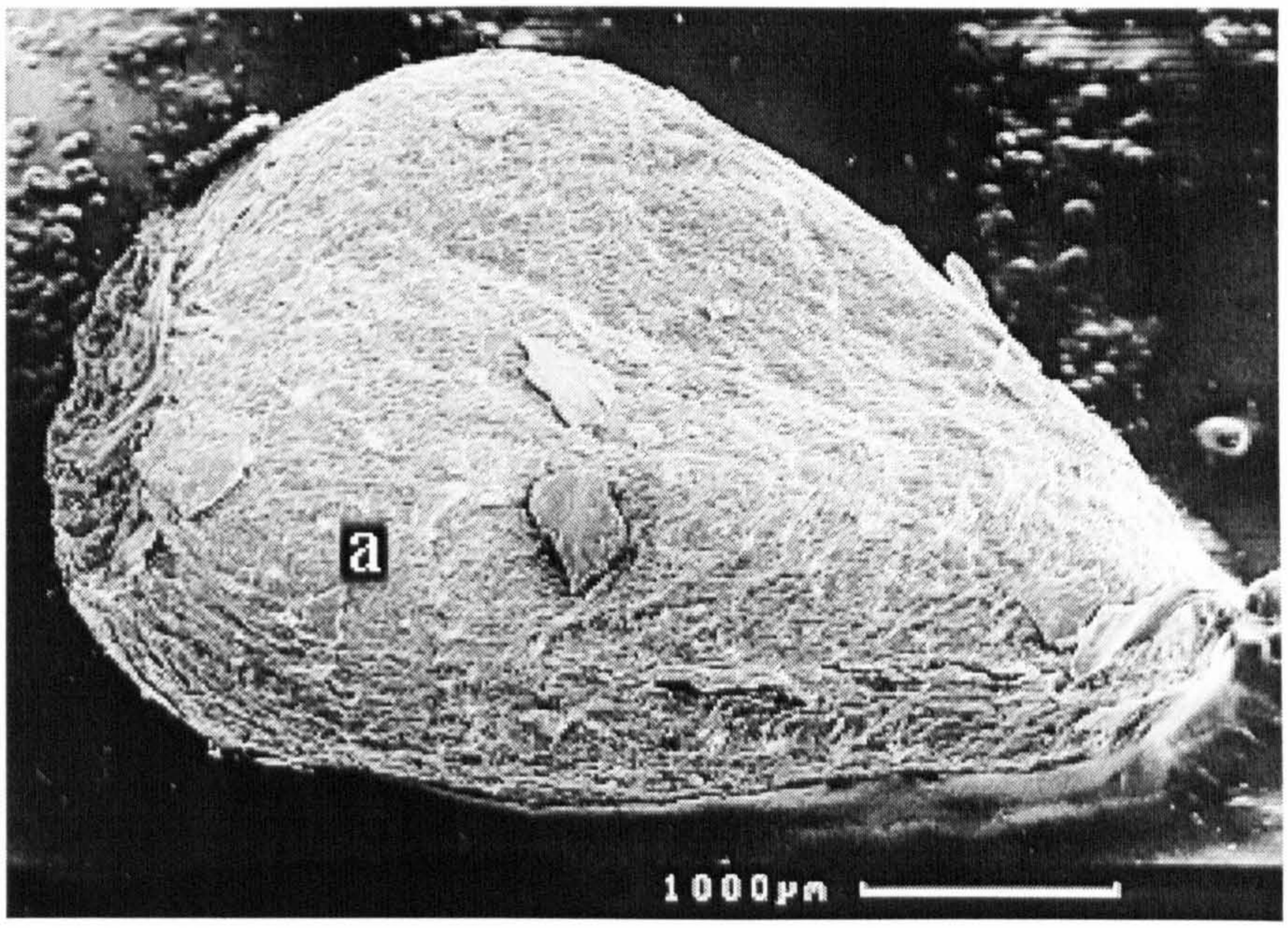


Plate 4.46 Commercially pretreated *Rosa corymbifera* 'Laxa' after 12 weeks in the warm period.

The seed coat surface has a few microbial structures associated with it (a).

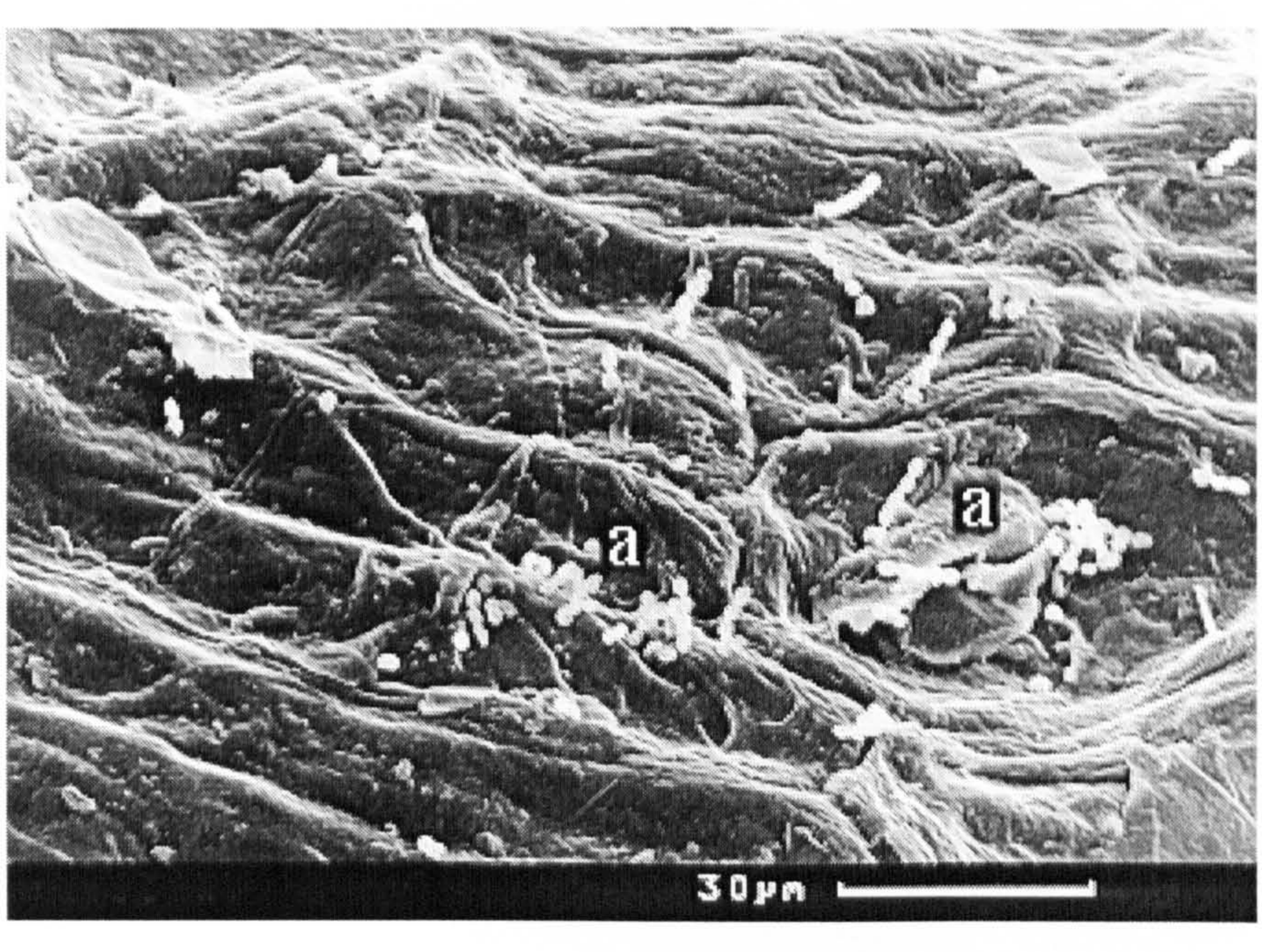


Plate 4.47 Fungal spores on the surface of the *Rosa corymbifera* 'Laxa' seed in plate 4.46. A few spores are visible at this higher magnification (a).

The Garotta pretreated seed sampled in week 12 is shown in plates 4.48 to 4.53.

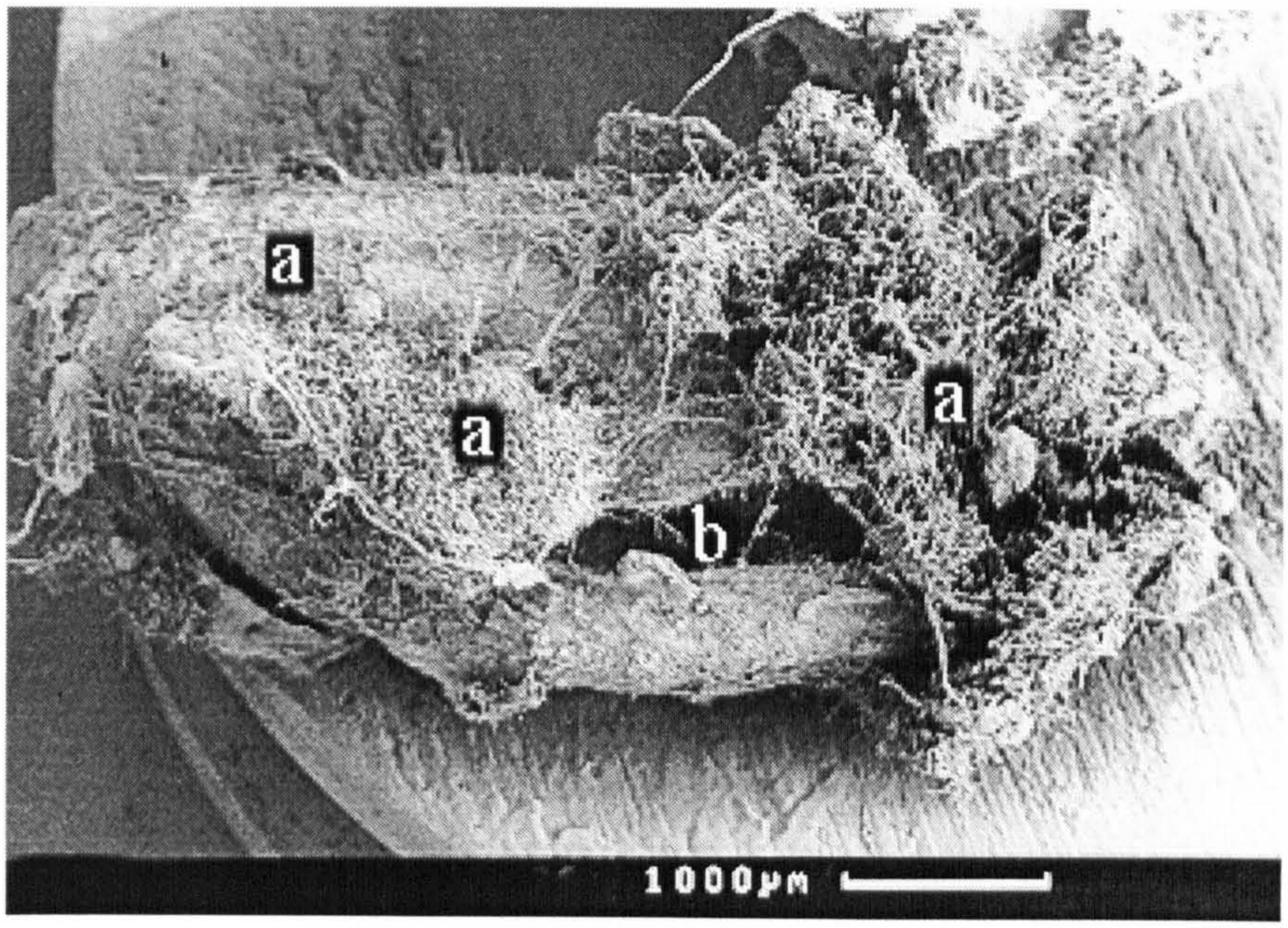


Plate 4.48 Garotta pretreated *Rosa corymbifera* 'Laxa' seed after 12 weeks at 25°C.
The coat is covered in fungal hyphae (a). As with most Garotta pretreated seed it has split open along the suture (b).

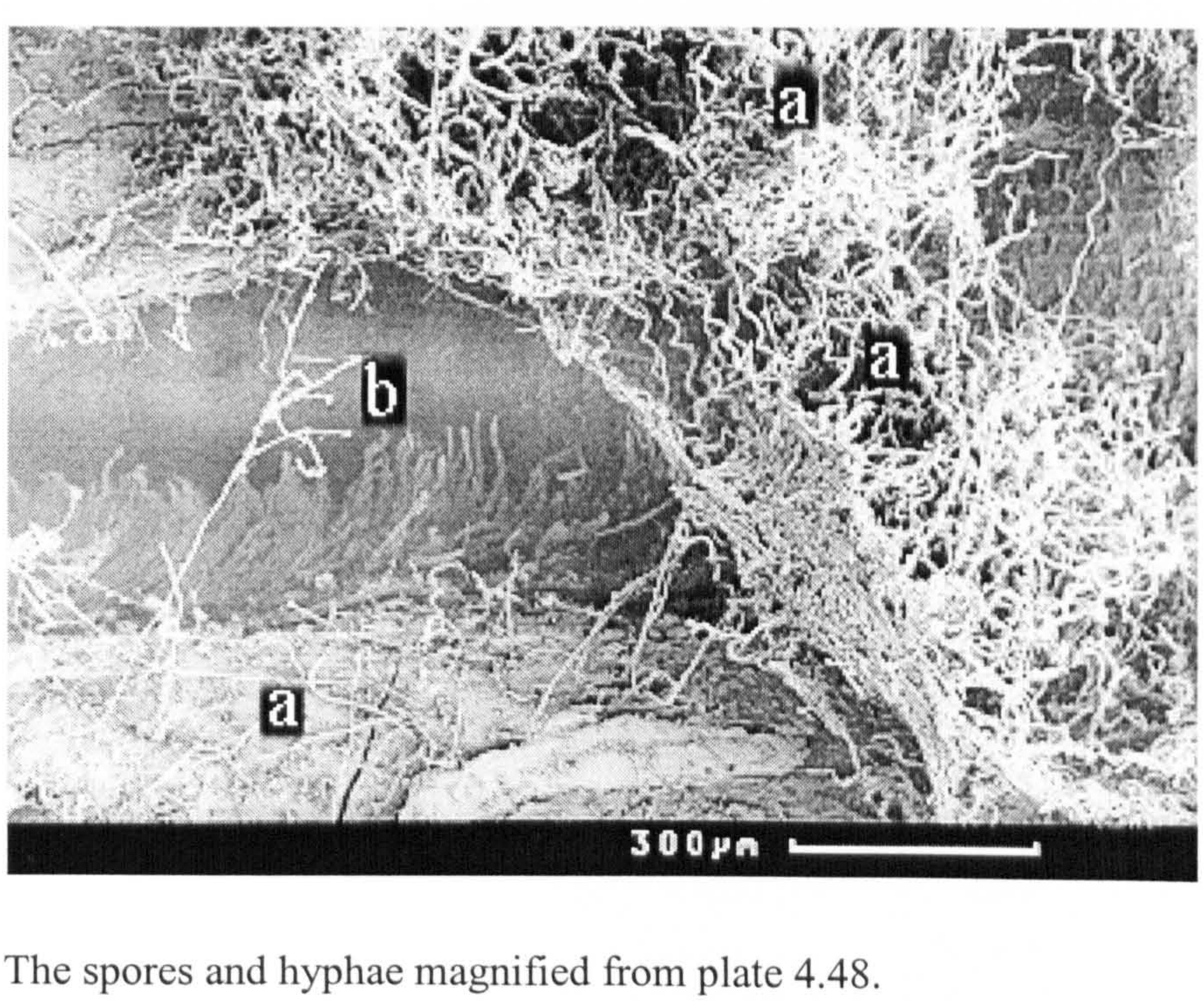


Plate 4.49 The spores and hyphae magnified from plate 4.48.
Whilst there is a dense covering of fungal hyphae intermingled with collapsed spores (a). The split suture reveals the embryo in the centre of the plate (b).

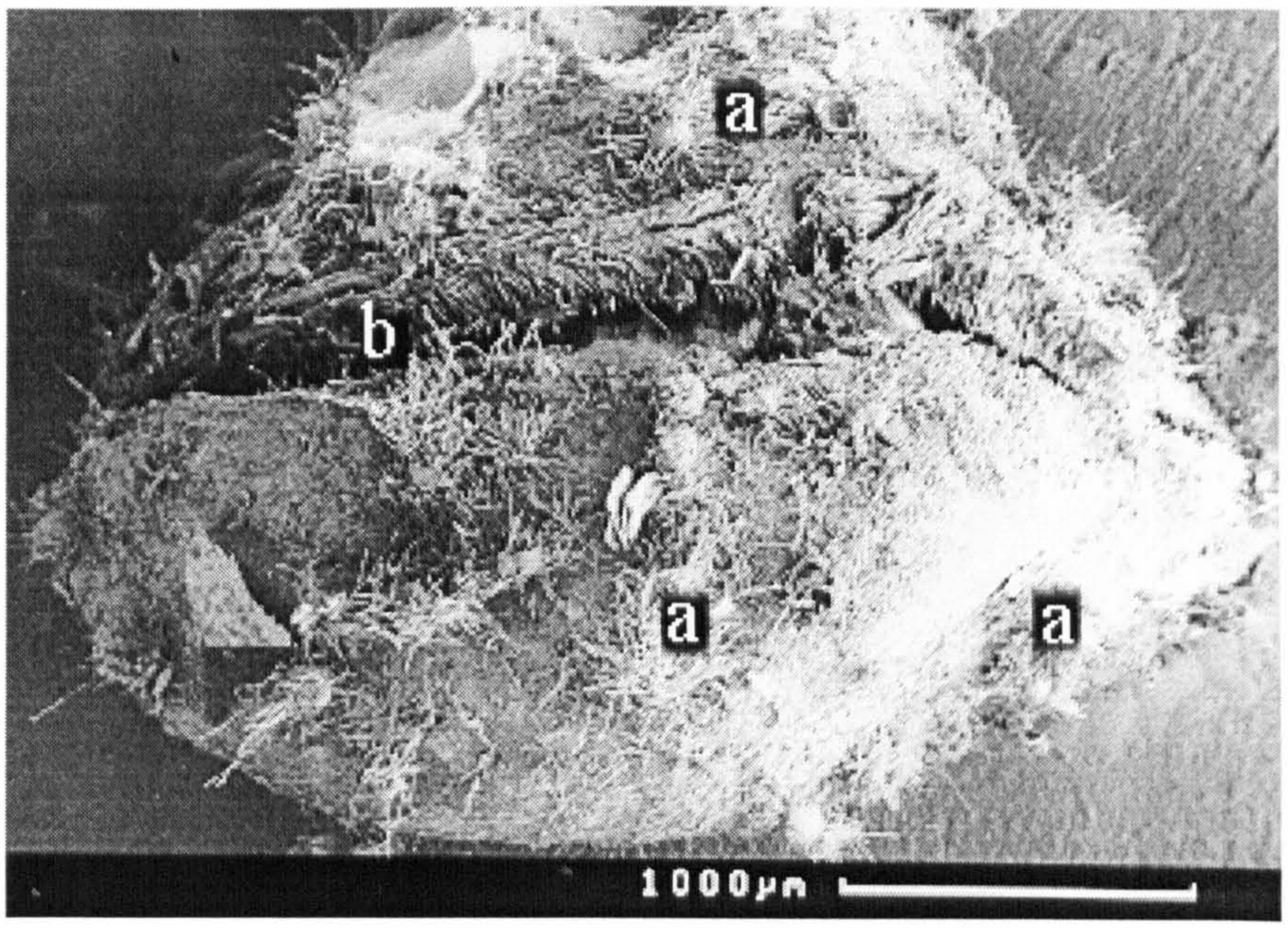


Plate 4.50 A *Rosa corymbifera* 'Laxa' seed, pretreated with Garotta, showing dense hyphal growth.
The fungal structures, hyphae and spores (a) (shown more clearly below, plate 4.51) do not look active and are likely to be remanents from previous growth. This seed is also split along the suture (b).

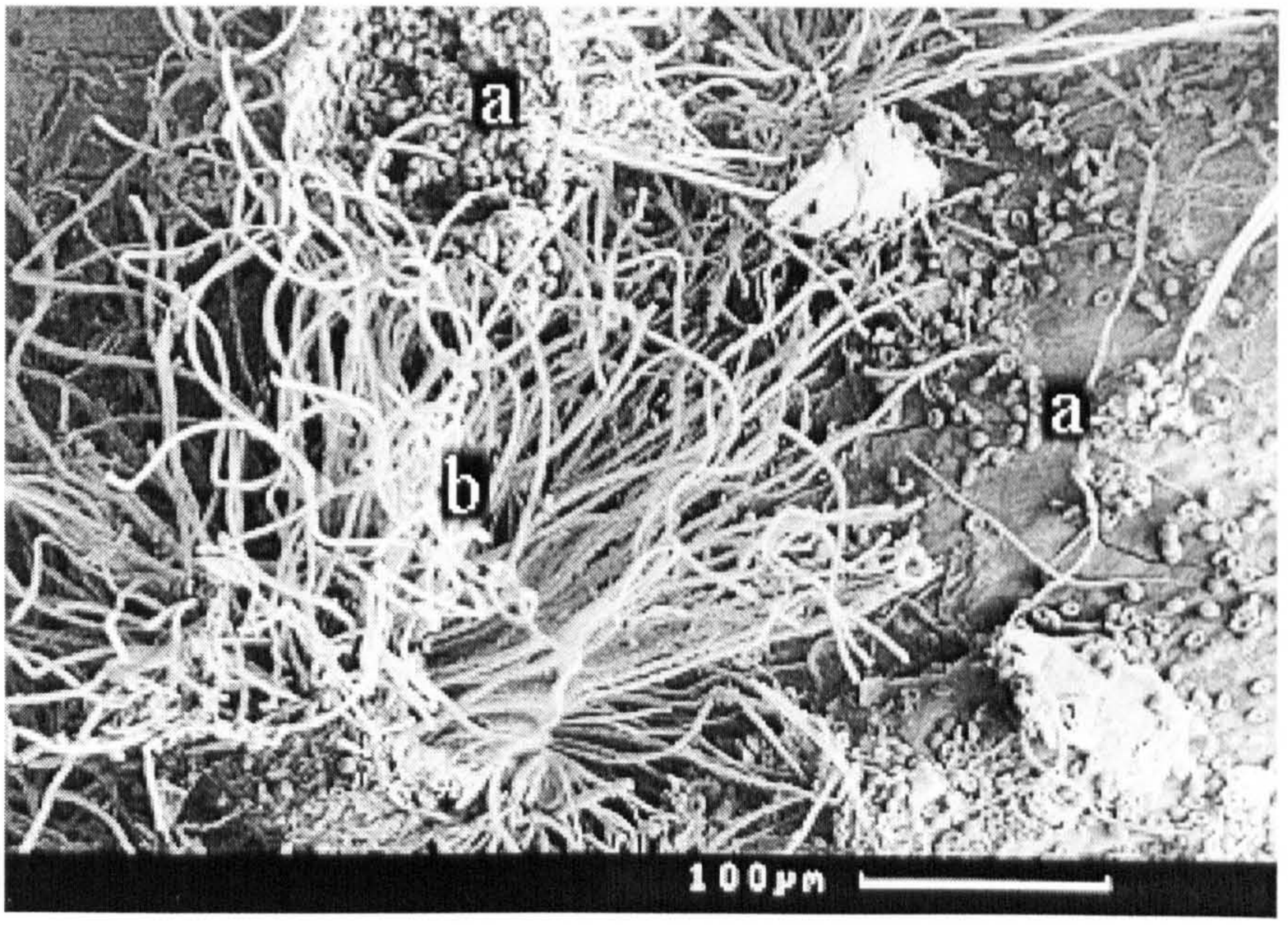


Plate 4.51 The fungal spores and hyphae from plate 4.50.
The spores have collapsed (a) (compared with those in plate 4.39, for example) and the hyphae are not actively growing (b) (compared to those in plate 4.36, for example).

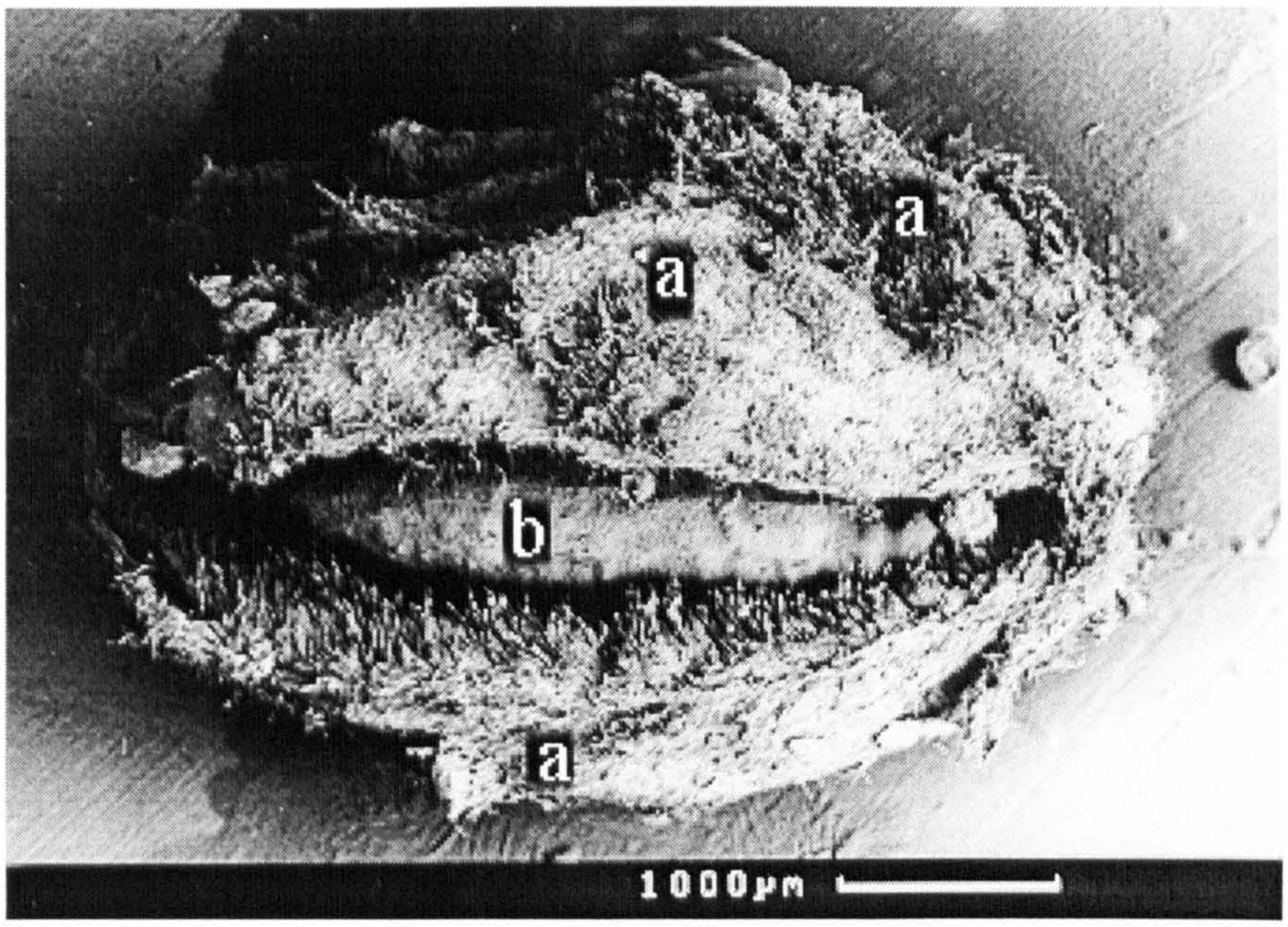


Plate 4.52 Patches of fungal growth on another Garotta pretreated seed.
Whilst these areas of fungal growth look dense, again they do not appear to be very active (a). Prior to the cold period most Garotta pretreated seed has split, as this example shows clearly (b).

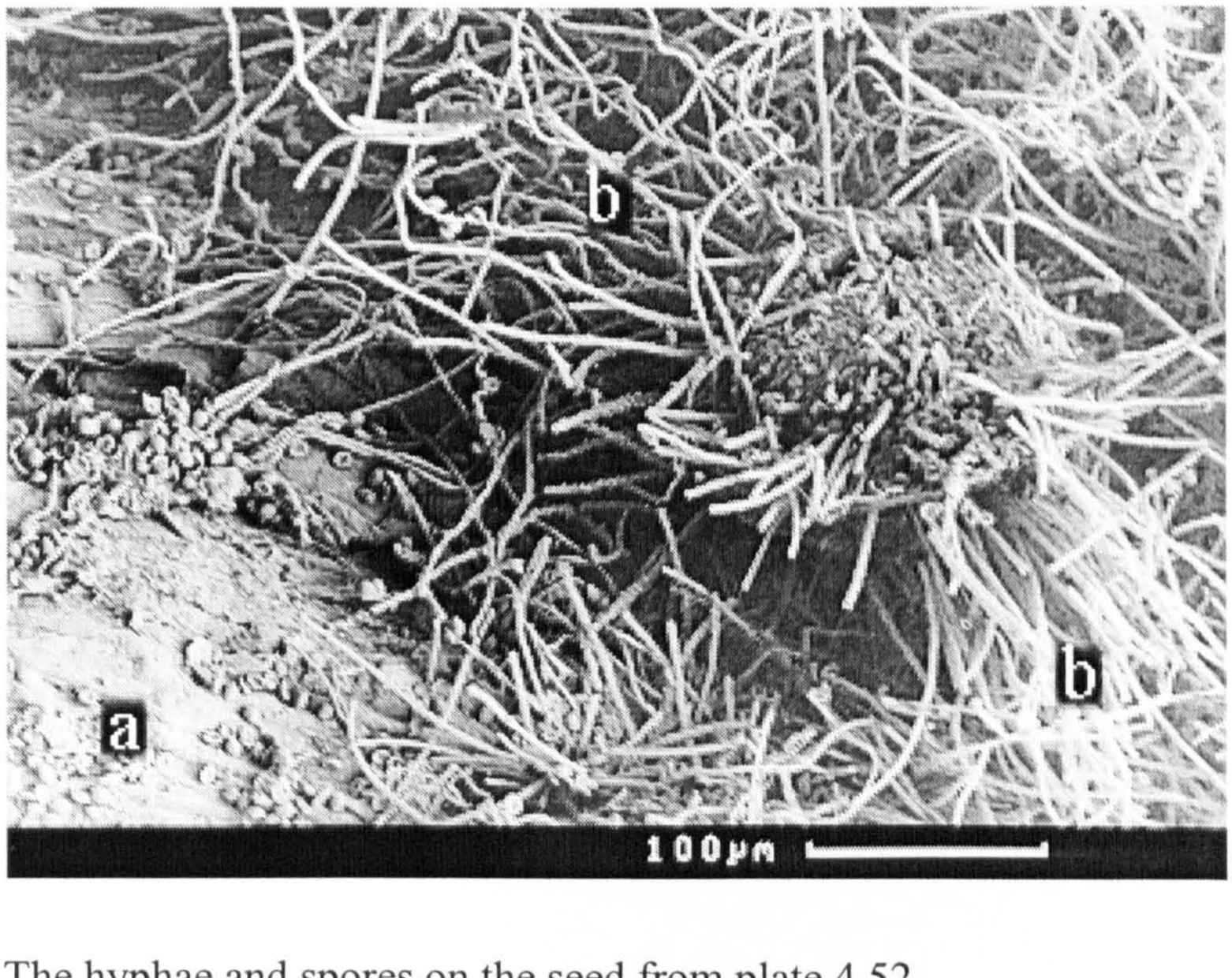


Plate 4.53 The hyphae and spores on the seed from plate 4.52.
The spores have collapsed (a) and the hyphae broken and fragmented (b). This is consistent with old fungal growth and is unlikely to be active.

4.4.2.4 Seed sampled after 14 weeks (2 weeks into cold period)

Commercially pretreated seed sampled in week 14 is shown in plates 4.54 to 4.59

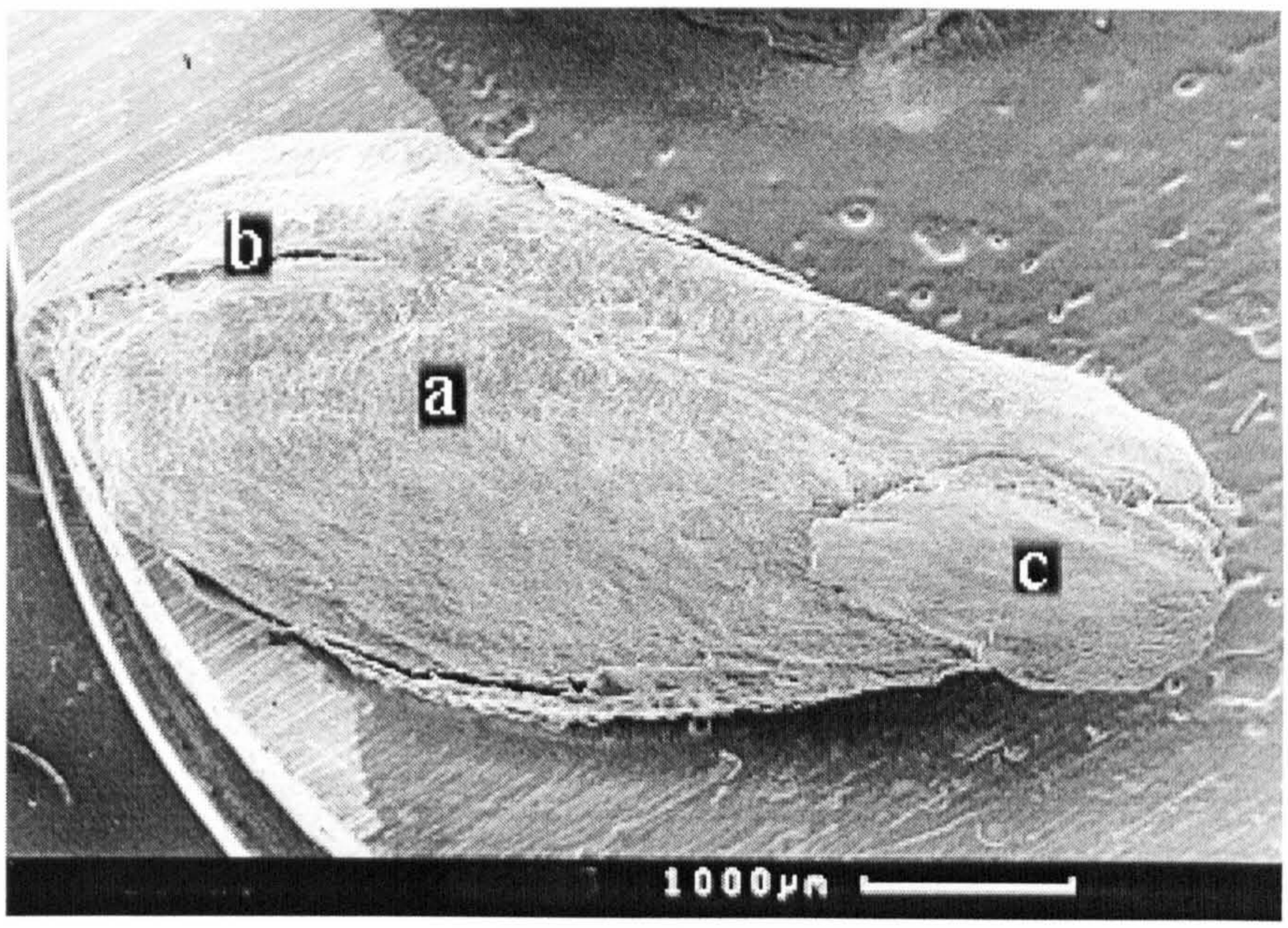


Plate 4.54 Commercially pretreated *Rosa corymbifera* 'Laxa' seed taken 2 weeks into the cold period.

The seed coat surface is very clean (a) with only a piece of vermiculite visible (c).

The suture has started to split (b), although this is likely to be due to drying rather than from the pretreatment.

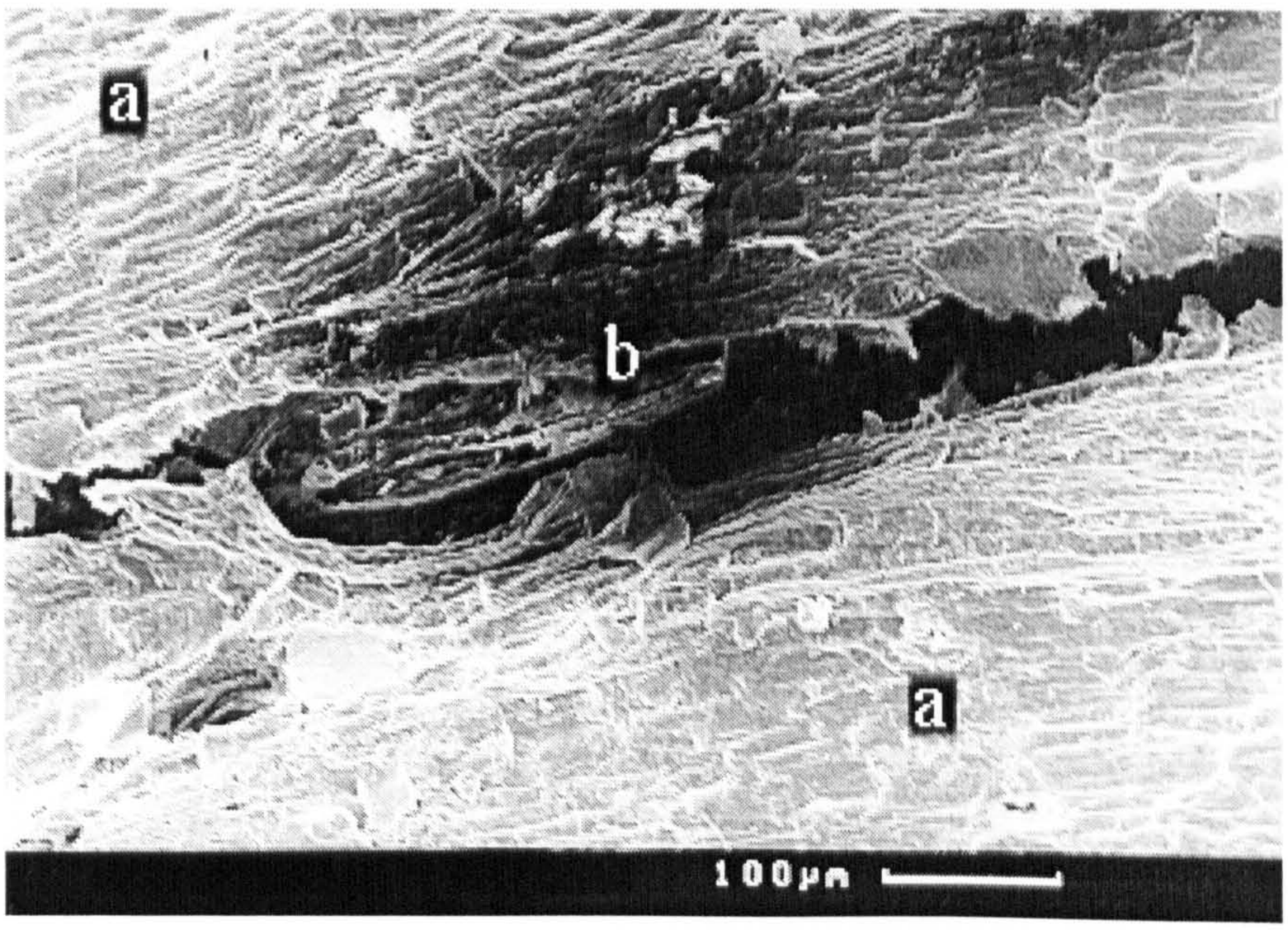


Plate 4.55 Magnified view of the apparent split in the seed coat from plate 4.54.

The surface shows no microbial structures (a), and unlike the Garotta pretreated equivalent seeds (plates 4.60, 4.62 and 4.64), no embryo is visible where the split has occurred (b). This confirms that the split is due to drying and not pretreatment.

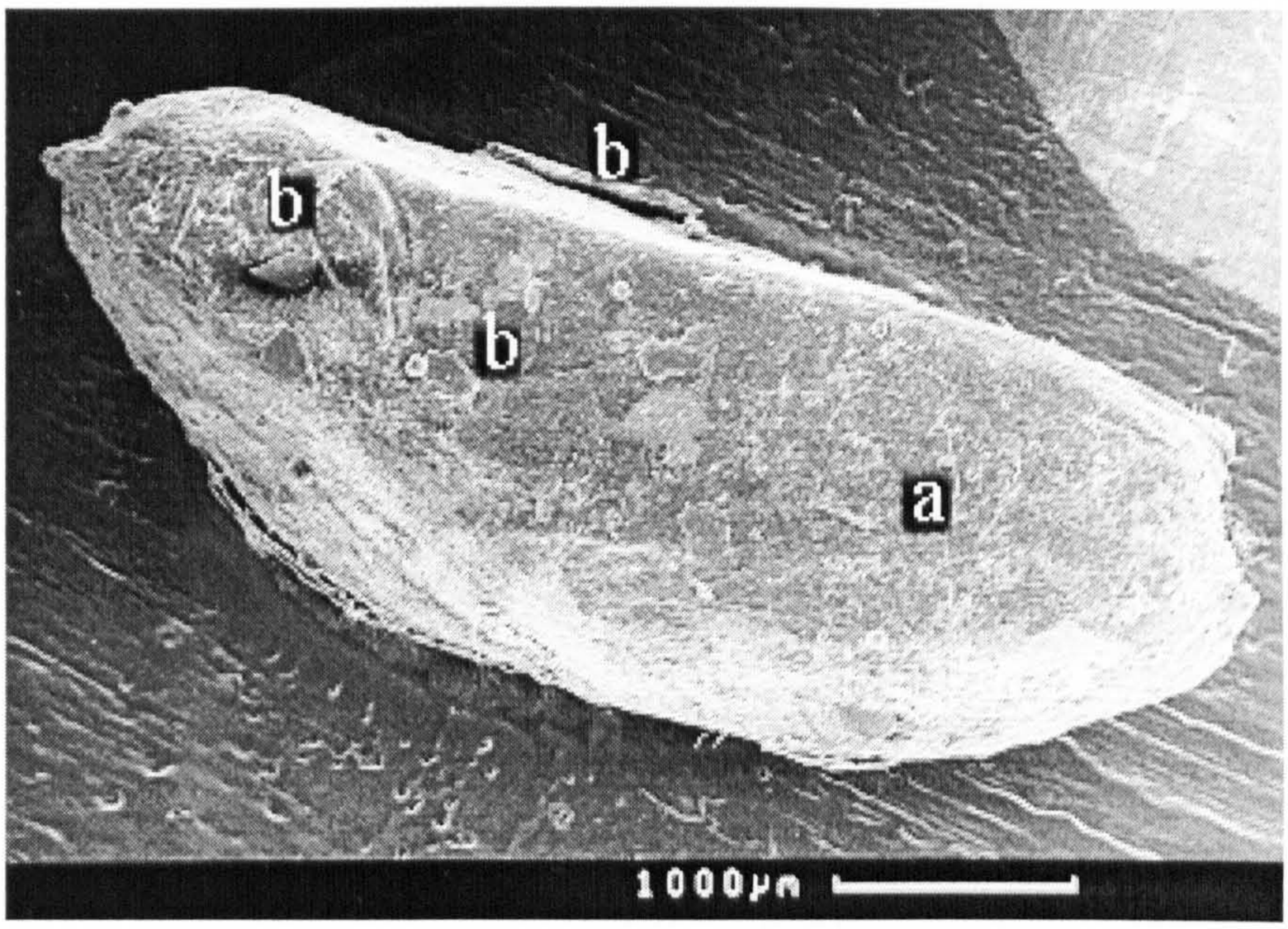


Plate 4.56 *Rosa corymbifera* 'Laxa' seed, commercially pretreated and sampled after 14 weeks.
The seed coat is very clean of any obvious microbial structures (a) and all that is visible is vermiculite (b).

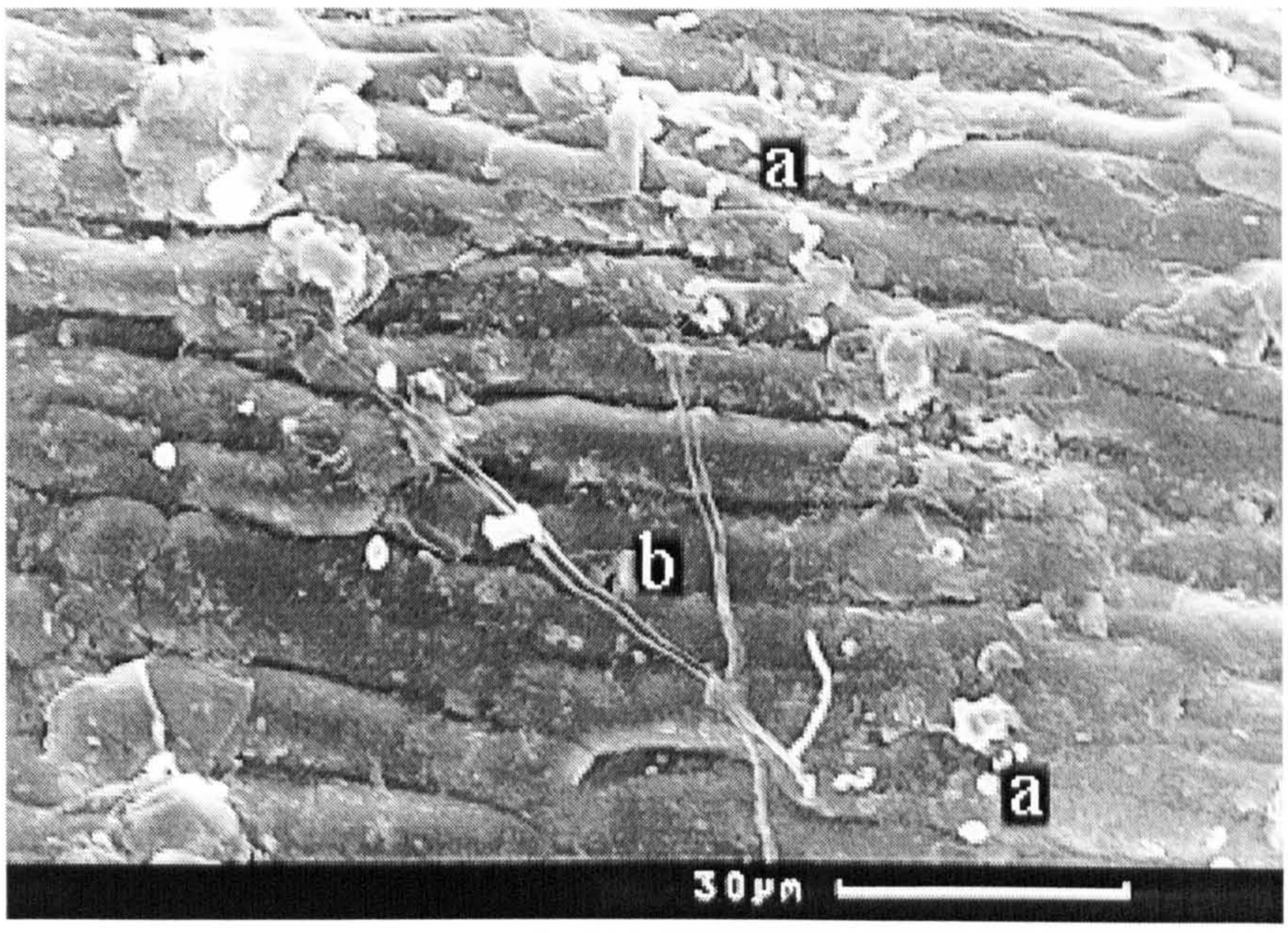


Plate 4.57 Fungal hyphae and spores on the seed coat surface.
Only a few spores were found (a) and the hyphae (b) had collapsed and were likely to be dead.

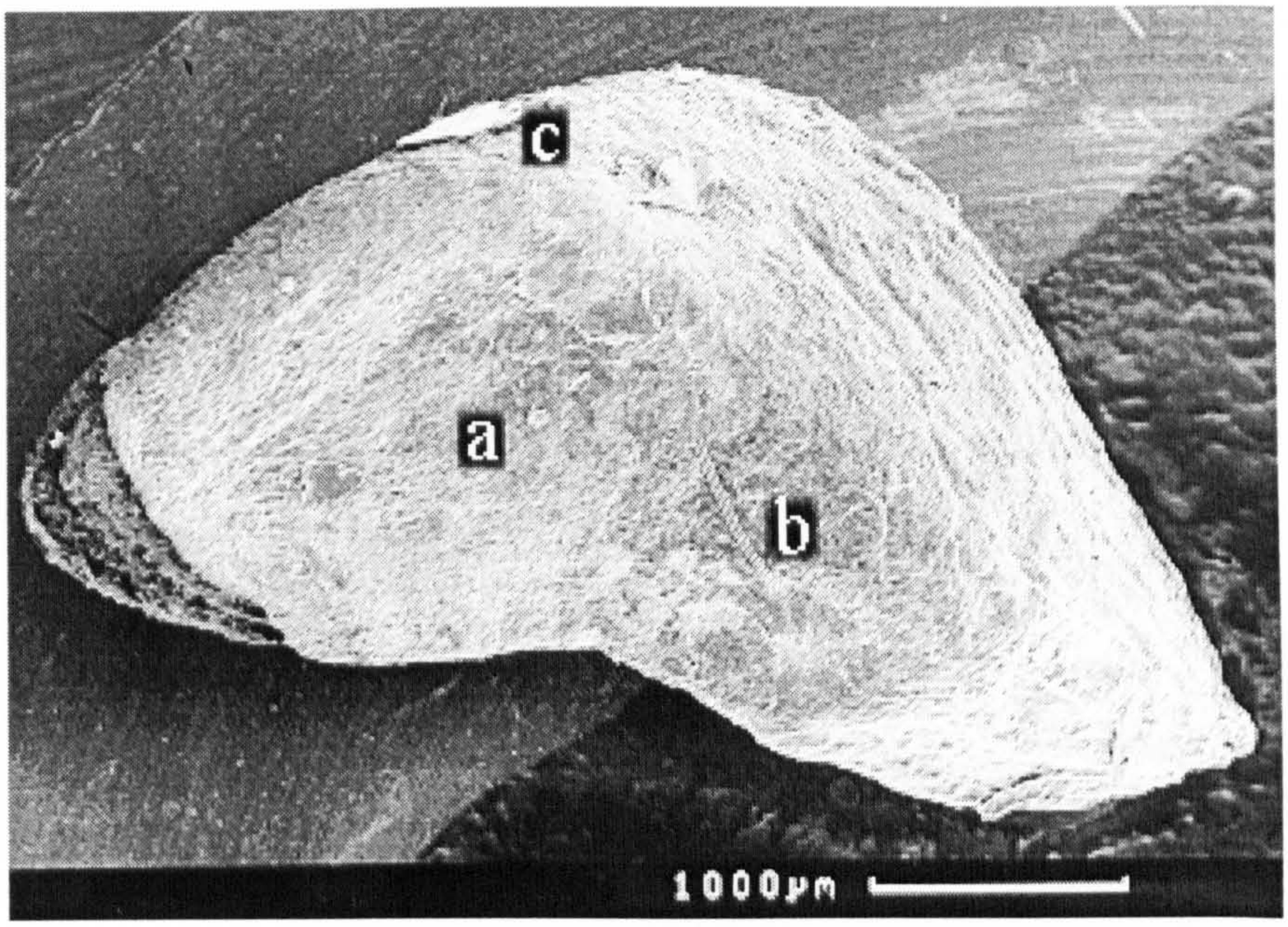


Plate 4.58 A third *Rosa corymbifera* 'Laxa' seed from the commercial pretreatment. The seed coat is again very clean (a). Vermiculite is present on the seed coat from the pretreatment mix (c), and a hair is still attached (b).

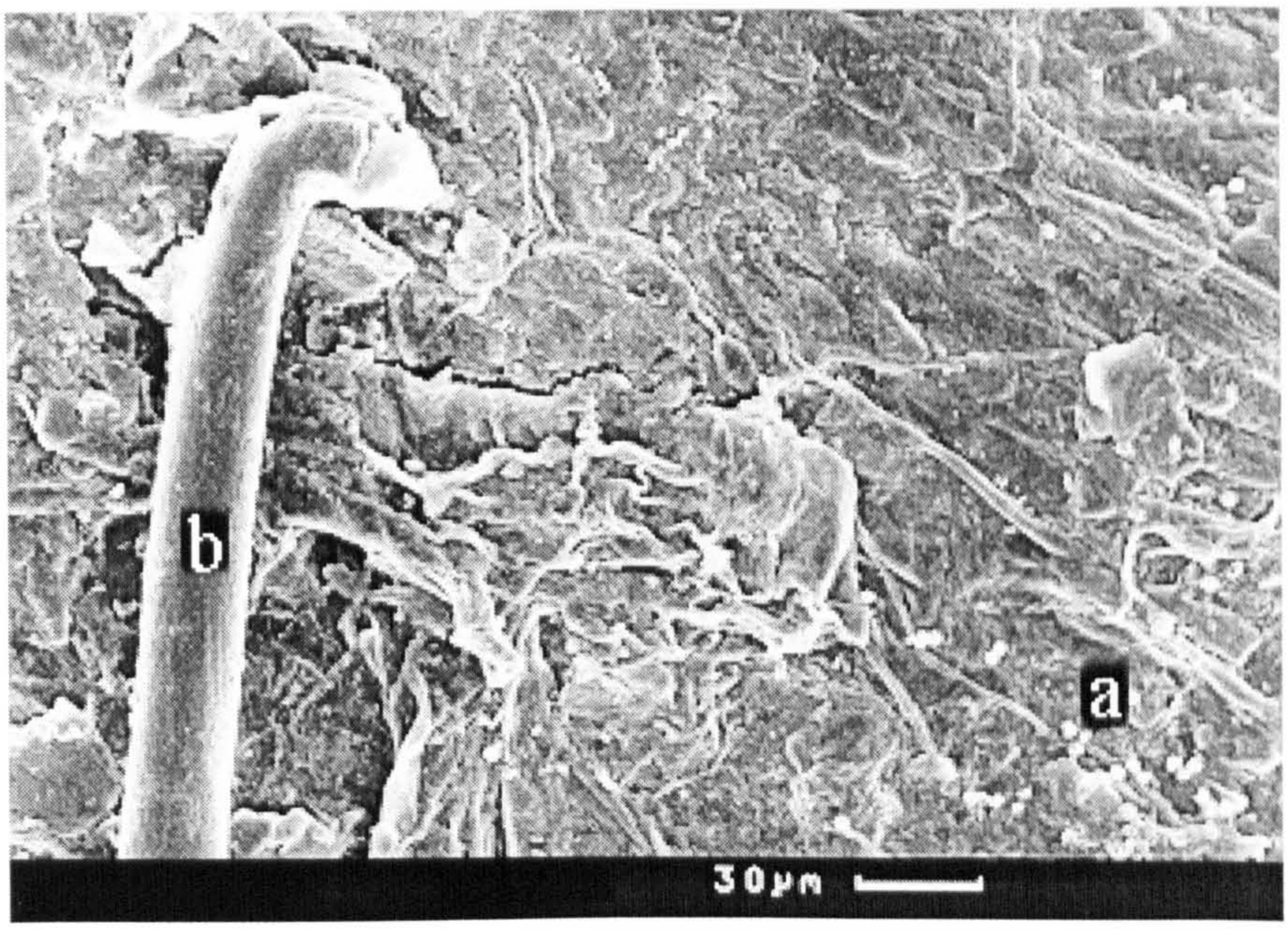


Plate 4.59 The seed coat surface magnified from plate 4.58.
A few spores are visible (a), although the majority of the seed coat is clean. The hair is clear to the left of the plate (b).

The Garotta pretreated seed from week 14 is shown in plates 4.60 to 4.65

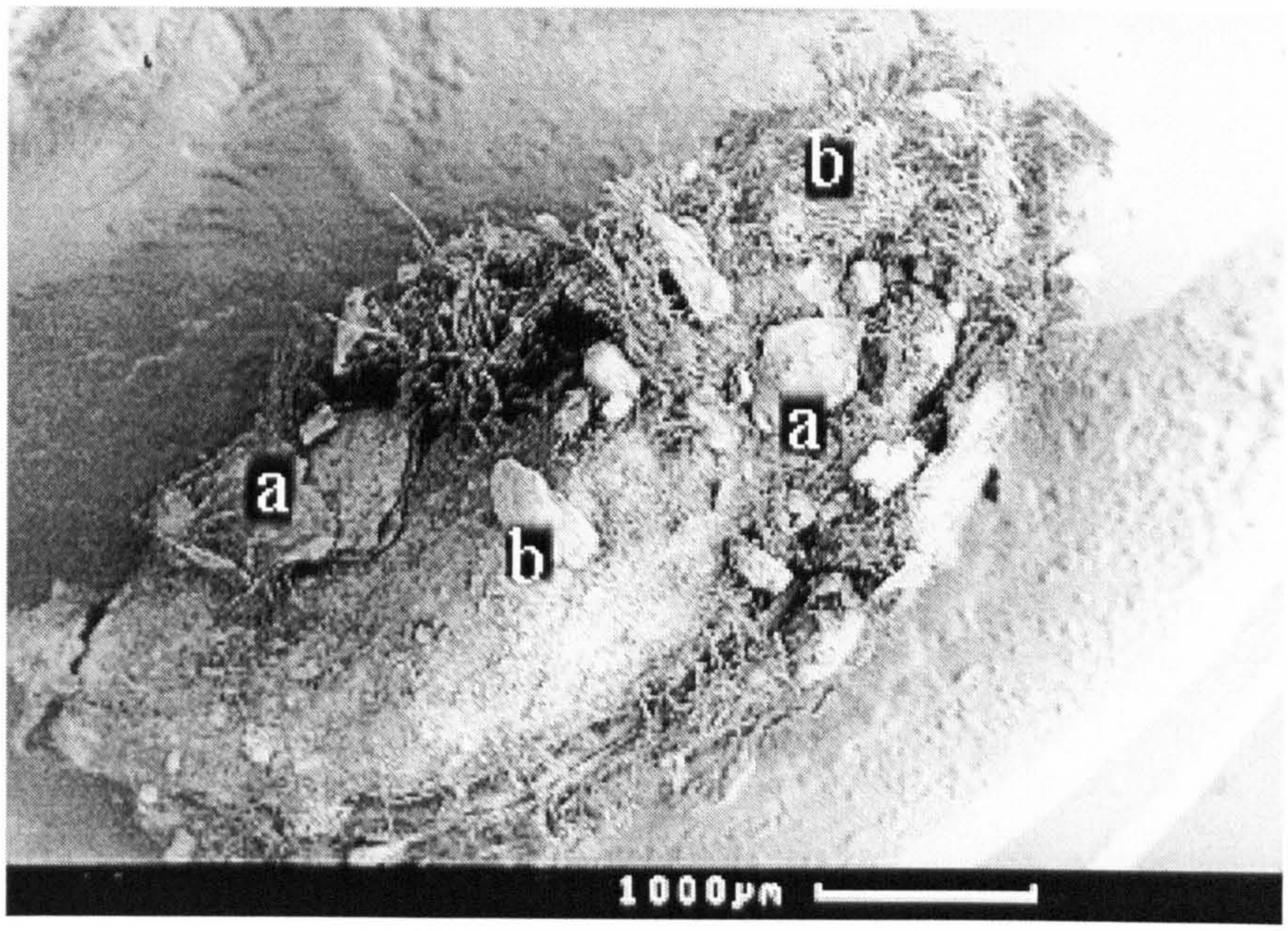


Plate 4.60 A Garotta pretreated *Rosa corymbifera* 'Laxa' seed, sampled from the pretreatment after 14 weeks (2 weeks into the cold period of incubation).
Dense areas of fungal growth are visible (a), along with pieces of vermiculite (b).

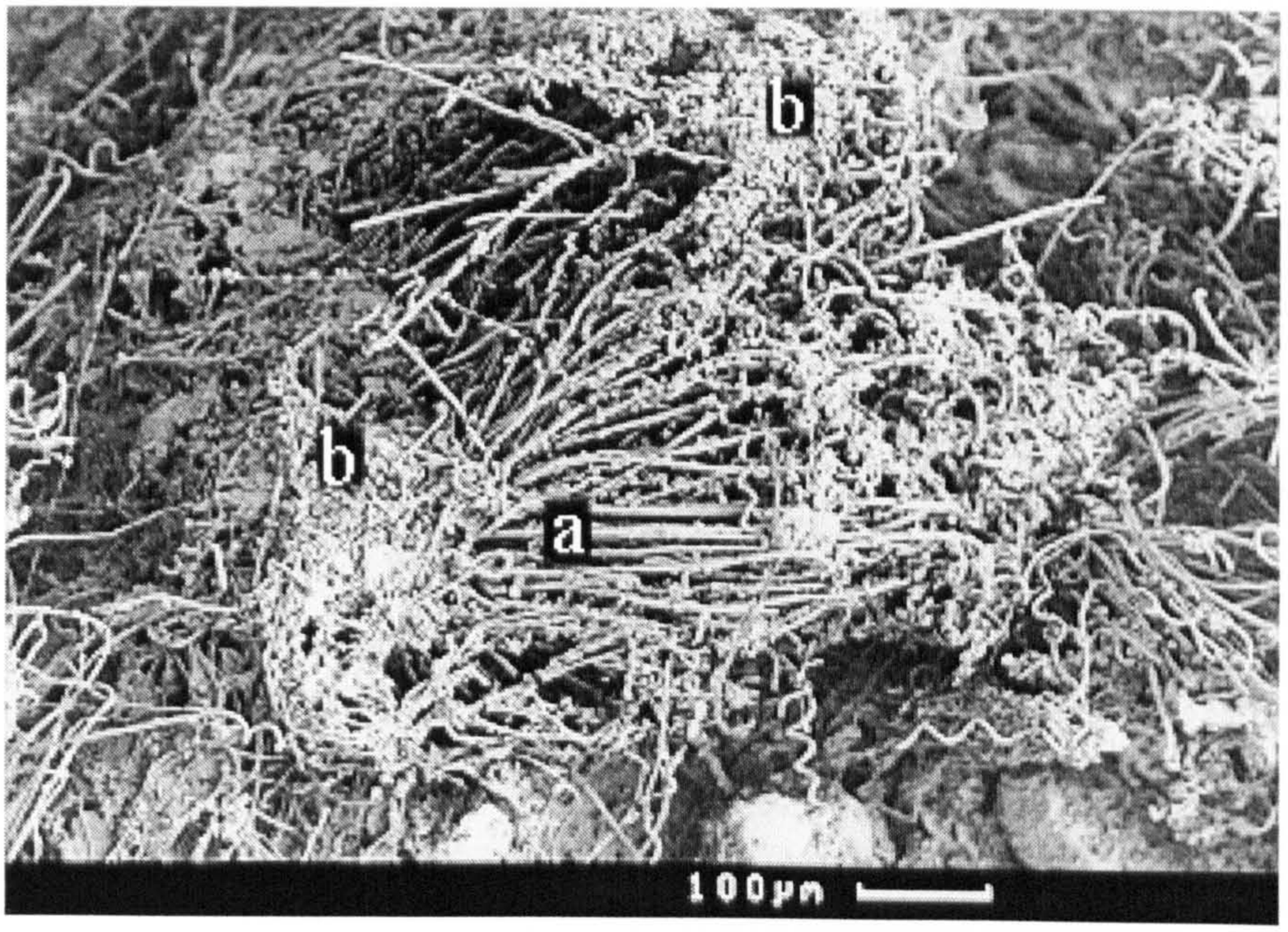


Plate 4.61 The fungal hyphae and spores magnified from plate 4.60.
The hyphae (a) and spores (b), whilst abundant, have collapsed and are not all likely to be viable.

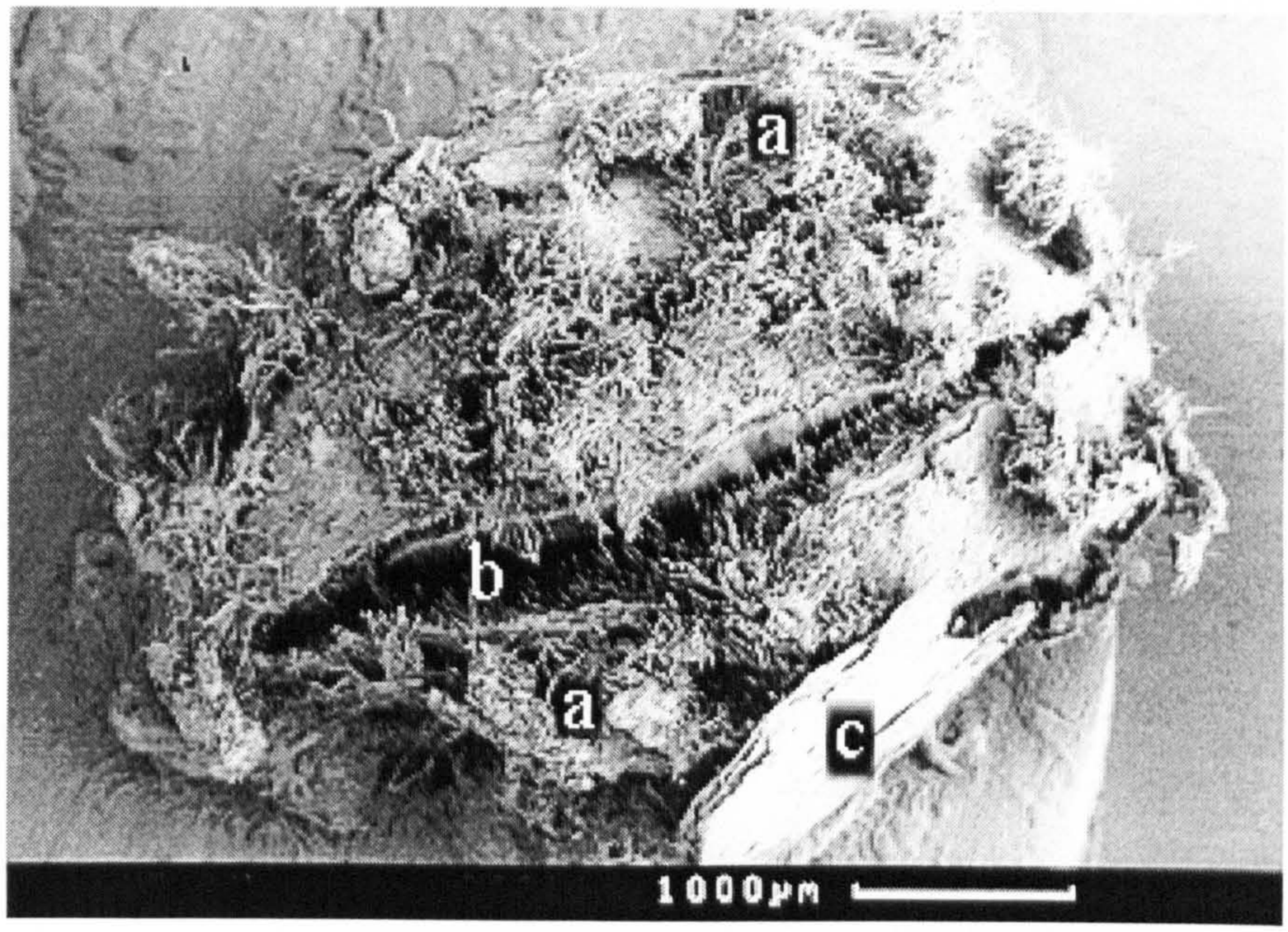


Plate 4.62 Dense fungal hyphae covering a Garotta pretreated seed.
Fungal hyphae are abundant on the seed coat (a). The characterisitc split suture of a Garotta pretreated seed is obvious (b), as is a large piece of vermiculite (c).

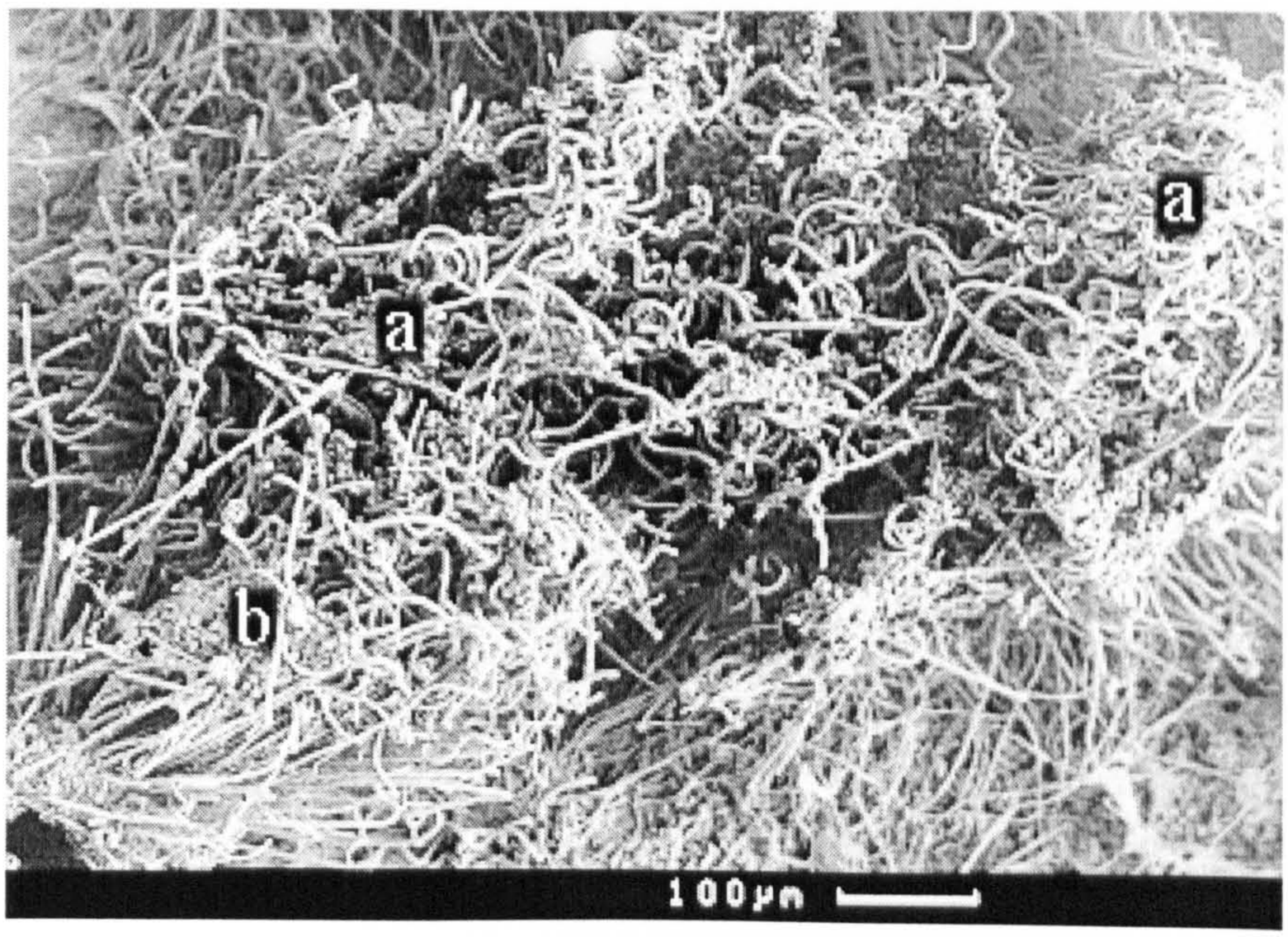


Plate 4.63 Fungal hyphae and spores on the coat of the seed in plate 4.62.
The hyphae (a) and the spores (b) all appear to be from old microbial growth which has ceased, hence their collapsed appearance.

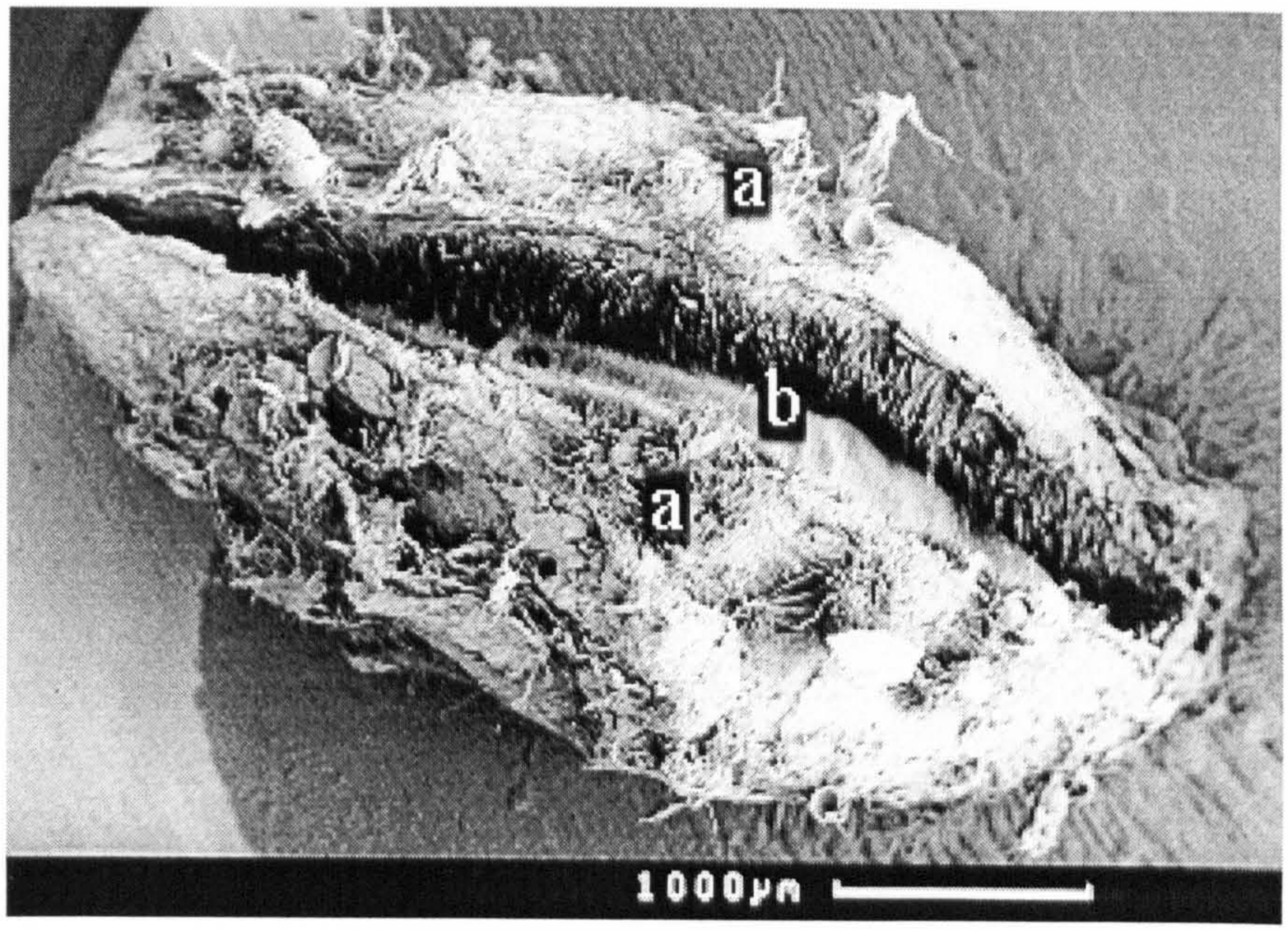


Plate 4.64 Garotta pretreated *Rosa corymbifera* 'Laxa' seed sampled after 14 weeks. The seed coat has many fungal growths over it (a). The embryo of the seed (b) is clearly visble through the split suture.

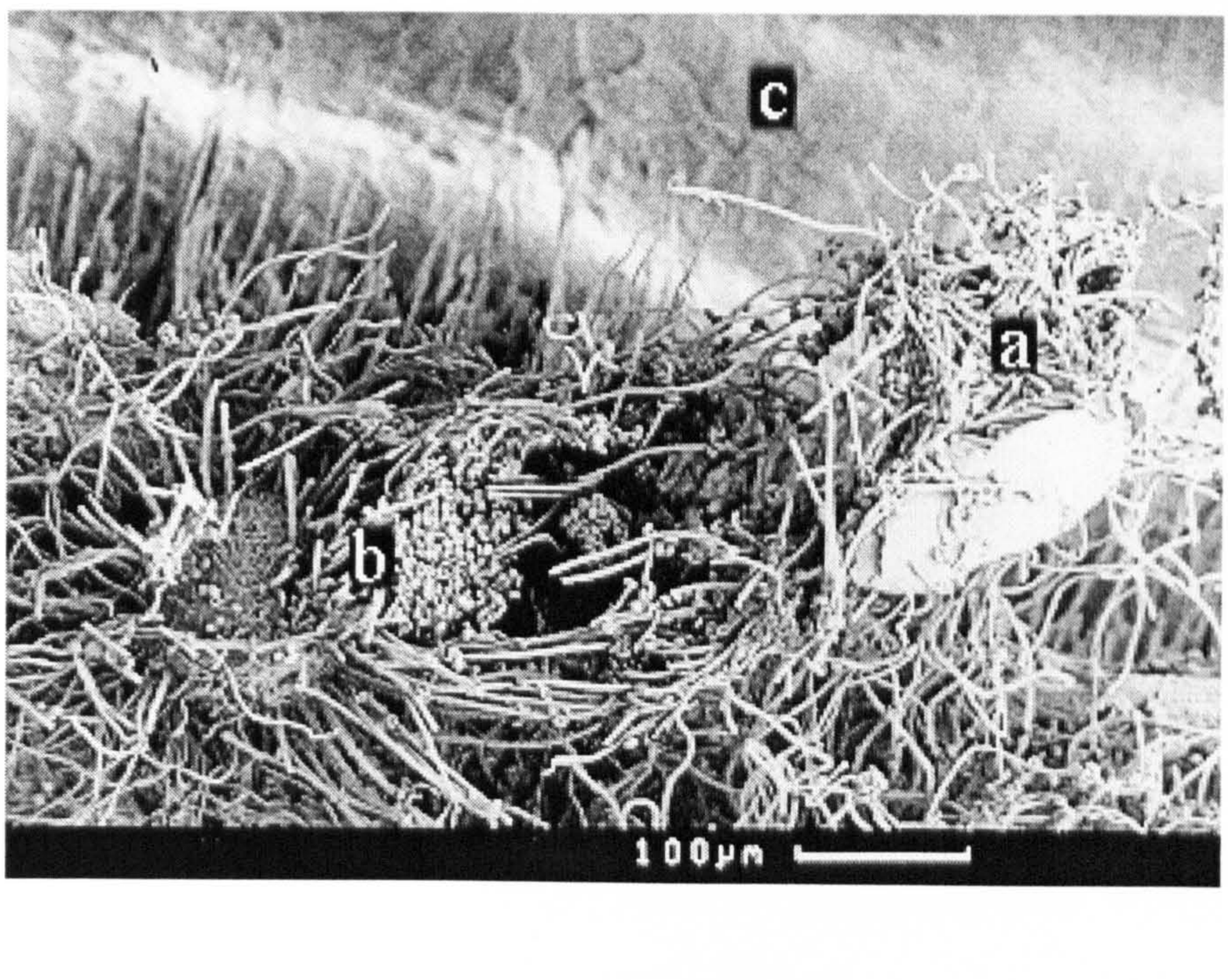


Plate 4.65 Higher magnification of the seed in plate 4.64.
The fungal hyphae (a) and the spores (b) are abundant, however, again they appear mainly collapsed and old. The embryo can be seen in the background (c) where the suture has split.

Seed was sampled after 3 weeks into the pretreatment as previous experiments had shown this to be the approximate time period when microbial growth and activity was at a peak (sections 4.1, 4.2 and 4.3).

The microbes associated with the commercially pretreated seed are shown in plates 4.19 - 4.26. Very few microbes were found on any of the seeds sampled. Those microbes that were found would have originated from the initial microbial loading identified on the seeds at setup (plates 4.7 - 4.12).

Plates 4.27 - 4.41 show the seeds sampled from the Garotta pretreatment. These are all well covered with microbes, in contrast to those seeds sampled from the commercially pretreated seed.

The *Rosa corymbifera* 'Laxa' seed sampled at weeks 12 and 14 showed similar patterns of microbial activity. All the seeds from the commercial pretreatment had few microbes associated with them. The seed coats were relatively clean and showed no signs of physical change. However, the Garotta pretreated seed was densly covered with fungal hyphae and spores. Close examination of these structures revealed much to be old and no longer active. The majority of what looked like dense microbial growth was in fact artefact from earlier on in the pretreatment.

4.4.3 Discussion

Very little evidence of microbes was found on seed of *Rosa corymbifera* 'Laxa' sampled at the setup of both pretreatments (plates 4.7 - 4.12). Close examination of the seed coats did reveal traces of microbes on all the seed. This correlates well with the low numbers initially found on untreated seed (section 2.2.2, microbial loading of seed). When samples of this seed were placed in a warm, humid environment for 48 hours, microbial growth was rapid and resulted in the seed being covered with microbes (plates 4.13 - 4.18). This demonstrates the great potential for microbial growth contained on these seeds.

The commercially pretreated and Garotta pretreated seed was all incubated under conditions conducive to microbial growth. However, after 3 weeks seed sampled from the commercial pretreatment showed little microbial activity. Initially this could be confusing, as the seed placed in a humid environment for 48 hours showed high coverage of microbes. However it is the timing that is the key. The commercial pretreatment contains no added nutrients, thus the microbes would initially grow in a similar pattern to those under the moist, humid environment. However, nutrients would soon become limiting thus restricting growth and eventually the microbes would die off.

The Garotta pretreatment on the other hand has a higher nutrient supply provided by the Garotta, and microbial growth is not restricted for the first 3 - 4 weeks. Thus the seed sampled after 3 weeks was densely covered with microbes. By the time the microbial numbers decline after 3 - 4 weeks, splitting of the seed is becoming obvious (the seed sampled at 3 weeks showed signs of splitting - plates 4.29 and 4.34)so further microbial action would have no benefit.

Seed sampled in the later stages of the pretreatments, i.e. 12 and 14 weeks, was still very similar in appearance to that sampled earlier. The commercially pretreated seed had very few signs of microbial growth, and the few spores and hyphae found may well have been residual left over from the very initial loading carried by the seed. The Garotta pretreated seed had dense patches of microbial growth, although most was likely to be inactive.

4.5 Enzyme Study

In light of the studies which showed that germination of *Rosa corymbifera* 'Laxa' was enhanced in the presence of microbial activity, it was decided to conduct a study of the effect of enzymes on the seed coat. It was hoped a similar splitting of the suture of the seed coat could be achieved as that found with the Garotta pretreatment after 4 weeks. Many companies were approached to try to obtain commercially available enzyme preparations with specific targets of plant tissues such as cellulose, lignin etc.

Commercial enzymes were used as, if successful, they would offer alternatives to Garotta at an affordable rate. Laboratory suppliers supply enzymes, however, it was not the purpose of the study to use these as they would be prohibitively expensive to a commercial seed company.

Objective - to use commercially available enzymes to try to degrade the seed coat.

4.5.1 Materials and Methods

Enzyme samples were kindly supplied by Rohm Enzyme Technology, Germany, Novo Nordisk Bioindustries U.K. Ltd, England and Courtalds Chemicals, England. A pure enzyme, digestase (isolated from deep sea crabs), was also used obtained from Professor Mike Trevan (South Bank University) which had been shown to have activity on many different substrates.

Preparations of all the enzymes were made according to recommended manufacturer's guidelines. Two ten fold dilutions were made to each side of the recommended concentration (based on the target substrate of the manufacturer). All enzymes were dissolved in sodium phosphate buffers of varying pH to optimise their performance, again based on recommendations. A table of the enzymes, their sources and their target substrates are shown overleaf (table 4.13).

25 ml of each each concentration of each enzyme was pipetted into separate 50ml conical flasks. Each had 25 *Rosa corymbifera* 'Laxa' seeds placed in the conical. These were sealed with parafilm and incubated in a shaking water bath at 25°C for 4 weeks. 5 seeds from each were taken each week and studied under the microscope for any physical changes. They

were then scraped and cut in two with a razor blade - 'cut test' - as a subjective test for any softening of the seed coat tissue. The control was sodium phosphate buffer as well as a separate distilled water control. A cocktail of all 11 enymes of each concentration was combined to also test the seed.

Table 4.13 The enymes used for study, their source and recommended target substrate.

Enzyme	Source	Target Substrate
Pectinex (pectinase)	Novo Nordisk	Soluble/insoluble pectins
Fungamyl (amylase)	Novo Nordisk	amylase
Alcalase (protease)	Novo Nordisk	protein
Celluclast (cellulase)	Novo Nordisk	cellulose
Rohalase (cellulases)	Rohm	cellulose/hemicellulose
Veron M3 (amylase)	Rohm	amylose
Veron GX (amylase)	Rohm	amylose
Rohapect (pectinase)	Rohm	pectin
Digestase (many enzymes)	Professor Mike Trevan	general
DP 1180 (cellulase)	Courtalds Chemicals	cellulose
DP 1204 (cellulase)	Courtalds Chemicals	cellulose

4.5.2 Results

No physical changes occurred to the seed in any of the enzyme preparations at any concentration. No seed coats appeared to soften and no seeds weakened noticably for the cut test.

4.5.3 Discussion

As no physical changes were detected for these experiments, no further work was carried out on the effect of commercial enzymes on the seed coats of *Rosa corymbifera* 'Laxa'. It would therefore appear that either the enzymes tested were either not specific enough for the seed coats or more likely a battery of enzymes would be required to break down the different components of the seed coat at the same time.

4.6 Summary discussion

The shapes of the curves obtained for the microbial counts in all pretreatments are similar to a standard microbial growth curve. In an enclosed system such as this there would be a lag phase before exponential growth, followed by a stationary phase. With the pretreatment of *Rosa corymbifera* 'Laxa', the lag phase was approximately 2 weeks, the exponential phase 2 weeks and the stationary effect was seen more as a death phase where microbial numbers could no longer be sustained. During the warm period of incubation fungal and bacterial numbers were always higher in the Garotta pretreatment than the commercial pretreatment.

The results of the microbial activity (FDA assay) and microbial population assessment (cotton strip assay) also produced curves very similar to a standard growth curve (as described above). This is not surprising as each measures a different aspect of microbial activity and will be related to the state of the microbes present at that particular time point. Peak activity in the FDA assay, cotton strip assay and microbial counts all coincided after 3 weeks (with the exception of week 7 in figure 4.8). The results showed greater microbial activity and microbial population for the Garotta pretreatment than the commercial pretreatment. Therefore cellulase activity was greater in the Garotta pretreatment.

The nutrient status of the pretreatments was paramount to the microbial population. The addition of Garotta was the only difference between the two pretreatments, and the microbial numbers, activity and population assessment were always much greater in the presence of it. In the absence of Garotta there was no nutrient source except that which the seed coat may provide. However, as previously discussed, wood (which is effectively the composition of the hard seed coat of *Rosa corymbifera* 'Laxa'), has a very high C/N ratio, most of which is immobile (Campbell, 1985). It is unlikely any nutrient would be available to the microbes.

With no nutrient source the microbes in the commercial pretreatment would have no way of growing and reproducing, hence numbers were very low, as was activity, population and visible growth. In the presence of Garotta the microbes could flourish, and this is reflected in the results obtained for the different assays. Interestingly, the addition of Garotta on a weekly basis did not affect germination percentage. The effect of the microbes has occurred after only 4 weeks (visible splitting of the seed coat) and even if microbial

numbers were higher when more Garotta was added, the 1.0g pretreatment is sufficient to produce the results consistently found. It would therefore appear that the addition of Garotta may stimulate the microorganisms by increasing the available nitrogen and thus greatly reducing the C/N ratio. Commercial enzymes showed no effect both on gross anatomy of the seed coat or on potential splitting of the suture.

The plates in section 4.4 show the succession of microbial growth on commercially pretreated and Garotta pretreated seed. The commercially pretreated seed never had dense microbial growth and only had traces of microbial structures present. Garotta pretreated seed however was densely covered with fungi from week 3 onward. The dense coverings found on seed sampled after weeks 12 and 14 were likely to be inactive, old structures. This correlates well with the findings of the microbial counts (section 4.1), FDA assay (section 4.2) and cotton strip assay (section 4.3) where the action of microbes diminished as the pretreatment continued, especially when the cold period was entered. Microbes would not actually die in the cold, but remain in a state of dormancy until temperatures increased (shown in section 4.1).

5. GERMINATION TRIALS

The culmination of any seed pretreatment is a germination test. This is to determine the maximum germination potential of a seed lot, which can in turn be used to compare the quality of different lots and pretreatments (ISTA, 1993). The usual method of testing is performed under laboratory conditions, and the result extrapolated to estimate the field planting density.

Germination can be defined in many different ways. However the general consensus is that germination is the resumption of active growth in the embryo of a seed, resulting in a seedling with the essential structures which would allow it to produce a satisfactory plant under favourable conditions in soil (ISTA, 1993; U.S. Department of Agriculture, 1974).

Whilst the germination test is used in this thesis as the ultimate criterion to determine the relative success of any given pretreatment, one particular experiment also used a viability test as an indicator of survival of *Rosa corymbifera* 'Laxa' seed during the pretreatment. This test was used as no seed germinated following the particular pretreatments used. It was essential to know therefore whether the embryos were still alive, thus an adapted excised embryo test was performed.

The excised embryo test for viability is specifically designed for seed which germinate slowly or show dormancy (ISTA, 1993). *Rosa corymbifera* 'Laxa' falls into this category and so is appropriate for the test. The viability test was used to measure the number of seeds with 'firm and fresh' embryos still inside following the germination test. Decayed or absent embryos do not count as viable.

Germination rates were always determined following the pretreatment of any species of tree seed. The majority of the work centered around *Rosa corymbifera* 'Laxa', however, five other native species were studied in one large scale field and laboratory trial to ascertain whether the Garotta pretreatment could enhance the germination of other species as well as *Rosa corymbifera* 'Laxa'. Thus, laboratory and field trials on germination were conducted on *Rosa corymbifera* 'Laxa', *Crataegus monogyna* (Hawthorn), *Rosa canina* (Wild Dog Rose), *Tilia platyphyllos* (Red-twigged lime), *Acer campestre* (Field Maple) and *Prunus spinosa* (Blackthorn).

As all these species are traditionally raised from seed outside in seedbeds, the importance of the field trial was obvious. It would also allow direct comparison to determine whether the laboratory germination tests were comparable to those obtained in the field trial.

This chapter is split into two sections, the laboratory germination studies and the field germination studies. In turn these two sections are further divided into the more detailed studies on *Rosa corymbifera* 'Laxa' and then the other five native species.

Aims - to show that native tree seed can be effectively germinated in the laboratory and the field, and to establish the effect on germination caused by the Garotta pretreatment compared to the commercial pretreatment.

Objectives - to show that the addition of Garotta to the pretreatment of native tree seeds enhances percentage germination.
To investigate whether two of the components of Garotta can act as substitutes during the pretreatment of *Rosa corymbifera* 'Laxa'.
To show whether it is the Garotta alone or whether it is the action of microbes which causes the enhanced germination of *Rosa corymbifera* 'Laxa'.
To show that after pretreatment with Garotta, percentage germination of *Rosa corymbifera* 'Laxa' was enhanced over the commercial pretreatment in the field.
To determine whether the laboratory and field germination percentages were comparable and to report whether the laboratory system was an acceptable and valid alternative to the field trial.

5.1 Laboratory germination trials

After the 24 week pretreatment period, samples of seed from all the species were taken for germination tests. Where possible the ISTA guidelines were used to determine quantities sampled and substrates used to germinate the seeds upon. Germination tests were carried out on all pretreatments when appropriate.

Based on the information from the studies using Garotta to enhance the germination of *Rosa corymbifera* 'Laxa', experiments were devised to ascertain whether any of the key components identified in the analysis of Garotta (section 2.3) and of particular importance to microbial growth would enhance germination of *Rosa corymbifera* 'Laxa'. The purpose was to substitute a particular compound, containing a known amount of the key component (section 2.3), for Garotta.

The studies investigating the pretreatment of *Rosa corymbifera* 'Laxa' have so far found that the addition of Garotta to the pretreatment results in enhanced germination when compared to the commercial pretreatment. Microbial growth and activity are also found to increase in the presence of Garotta, and the implication that the microbes cause the enhancement in germination is a strong one. However, it is possible that it is a chemical effect caused by the Garotta which enhances germination.

Objectives - to show that the addition of Garotta to the pretreatment of native tree seeds enhances percentage germination
To investigate whether two of the components of Garotta can act as substitutes during the pretreatment of *Rosa corymbifera* 'Laxa'
To show whether it is the Garotta alone or whether it is the action of microbes which causes the enhanced germination of *Rosa corymbifera* 'Laxa'.

5.1.1 *Rosa corymbifera* 'Laxa' germination trials

Samples of *Rosa corymbifera* 'Laxa' seed were subjected to various pretreatments to ascertain what was causing the enhancement in germination already seen. Thus the 'normal' pretreatments were used - commercial pretreatment, 0.5g Garotta pretreatment and the 1.0g Garotta pretreatment.

Pretreatment experiments were run with two of the main components of Garotta to see if a chemical substitute could be used. The 1.0g Garotta pretreatment and commercial pretreatment were run as 'controls'. These pretreatments were also run with surface sterilised seed to eliminate the microbial loading present on *Rosa corymbifera* 'Laxa' seed.

An experiment was also run adding Garotta at weekly intervals to the pretreatment to ascertain whether it was a limiting factor to germination. The amount of Garotta added each week was the equivalent to the 1.0g rate used at the setup of the 1.0g Garotta pretreatment. The pretreatments in this experiment were incubated in the warm for 6 weeks (instead of the ususal 12) and 12 weeks in the cold (as with previous pretreatments). This was to see if the pretreatment time could be reduced. The 'normal' 12 week warm and 12 week cold incubation was run concurrently with the 'normal' 1.0g Garotta pretreatment and commercial pretreatment.

5.1.1.1 Materials and Methods

Laboratory germination tests for *Rosa corymbifera* 'Laxa' were carried out using the sample size dictated by the ISTA guidelines (1993). Four lots of 100 seeds were removed from each replicate of each pretreatment and placed on moist filter paper set in petri dishes. Distilled water was added, and the samples incubated at 22°C. Four replicates were used for each pretreatment. Germination counts were made after 7, 14 and 21 days after removal from the pretreatments.

The pretreatments assessed were the commercial pretreatment, 0.5g Garotta pretreatment and 1.0g Garotta pretreatment.

Two key components were identified to use as substitutes for Garotta. They were selected on the basis of the high levels of those particular elements found in Garotta and important for microbial growth. They were phosphorus (phosphate) and nitrogen (nitrate).

The compounds used were Gem Superphosphate (P_2O_5) and Nitram (NH_4NO_3). The criteria for choosing these particular compounds were that they provided only the particular nutrient required, that they are both water soluble and both readily available. Each was used as separate pretreatments, and also combined together. The commercial pretreatment and 1.0g Garotta pretreatments were run for comparison.

The same five pretreatments were used as described above (i.e. the commercial, 1.0g Garotta, Nitram, Gem Superphosphate and a mixture of Nitram and Gem Superphosphate pretreatments). However in this case seed was surface sterilised to eliminate the microbial loading associated with the seed coat.

Rosa corymbifera 'Laxa' seed for all pretreatments was soaked for 24 hours in sterile tap water. Surface sterilised seed was prepared by taking the seed which had been soaked for 24 hours and agitating it for 20 minutes in 2% v/v detergent. The detergent was rinsed away using three washings of sterile distilled water. The seed was then agitated for 5 minutes in excess 70% ethanol and again rinsed three times with distilled water. Excess 2% w/v sodium chloride solution was added to the seed and agitated for 20 minutes and then rinsed three times with sterile distilled water. Seed was then ready to be used in the pretreatments.

25g moist vermiculite was placed into a $50cm^3$ conical flask. This was then stoppered with a foam bung and aluminium foil and autoclaved. Using aseptic techniques 10g of the surface sterilised seed was placed into each conical flask with the appropriate addition of Garotta or other compound (previously sterilised for 30 minutes under uv light). The conical flasks were then incubated at 25°C for 12 weeks and 4°C for a further 12 weeks.

Unsterilised seed was manipulated under similar conditions into the conical flasks. The results for the unsterilised seed were presented in the previous section, (5.3). 10 replicates were set up for each pretreatment, making a total of 100 conical flasks.

Sampling of seed occurred at setup, and of the pretreatment mix after 3, 6, 12 and 24 weeks. Two replicates per pretreatment were selected at random at each time point and samples washed and plated onto Potato Dextrose Agar and Nutrient Agar to check for microbes. Actual microbial counts were not made as only information regarding whether the pretreatments remained sterile was required. These replicates were then discarded in case any contamination occurred during the sampling. Seed was examined for any changes in physical apperance at each sample time.

After the germination counts were made, viability was measured of the remaining *Rosa corymbifera* 'Laxa' seed which had not germinated. This was a physical assessment based on cutting each seed open and assessing the state of the embryo. Healthy, viable embryos are white and plump. Dead embryos are dark brown and shrivelled. The results are expressed as a percentage of the viable seeds out of the total number of seeds remaining ungerminated.

At the end of the 24 week pretreatment incubation, 2 replicates remained per pretreatment. If all the previous replicates were found to be sterile, it was assumed that the last 2 would also be sterile. Equally, for the unsterilised seed pretreatments, where microbes were recorded throughout the pretreatment, it was assumed that these pretreatments underwent a 'typical' *Rosa corymbifera* 'Laxa' pretreatment as discussed in earlier chapters and sections.

Germination counts were performed on 4 lots of 100 seeds per replicate for each pretreatment. Viability was also assessed on the same criteria as described earlier in this section.

The weekly addition of Garotta to the pretreatment as well as the 1.0g Garotta pretreatment and commercial preteatment were incubated for 6 weeks at 25°C and 12 weeks at 4°C. The usual 12 weeks at 25°C and 12 weeks at 4°C pretreatments were also run for the 1.0g Garotta pretreatment and commercial pretreatment to allow direct comparison between the reduced pretreatment time.

5.1.1.2 Results

The results of the laboratory germination of *Rosa corymbifera* 'Laxa' are shown in table 5.1. It can be clearly seen that the addition of Garotta vastly increased the percentage germination of *Rosa corymbifera* 'Laxa' compared to the commercial pretreatment (81% and 93 % compared to 10%). However there was not such a marked difference in germination percentage when comparing the two different Garotta pretreatments (81% and 93%).

Table 5.1 Percentage germination for the laboratory studies on *Rosa corymbifera* 'Laxa' after 3 weeks.

	percentage germination*	SE
commercial	10.3	2.10
0.5g Garotta	81.0	1.91
1.0g Garotta	92.8	3.54

* average of four replicates of 100 seeds per pretreatment (i.e. 400 seeds per pretreatment).

Statistical analysis of the germination results found that there was a significant difference (0.1%) between the 0.5g Garotta pretreatment and commercial pretreatment, and also between the 1.0g Garotta pretreatment and commercial pretreatment. There was a significant difference (5%) between the germination percentages of the 0.5g Garotta pretreatment and 1.0g Garotta pretreatment. All raw data can be found in appendix 5.1.

The germination percentages for *Rosa corymbifera* 'Laxa' pretreated with the different compounds are shown in table 5.2. The commercial and 1.0g Garotta pretreatments were carried out to allow direct comparison of results. Also shown in this table are the viability percentages.

The Nitram (nitrogen source) and Gem superphosphate (phosphate source) on their own showed no enhancement of the germination of *Rosa corymbifera* 'Laxa' compared to the commercial pretreatment. However, when the two compounds were combined, percentage germination reached 29%. This was a great increase on the commercial pretreatment (2%) but fell well short of the level of germination obtained with the Garotta pretreatment (95%). All raw data can be found in appendix 5.1.

Table 5.2 Percentage germination and viability of *Rosa corymbifera* 'Laxa' seed following the various pretreatments.

	germination* (%)	SE	viability* (%)
Commercial	2.0	0.58	100
1.0g Garotta	95.2	0.40	100
Nitram	4.0	1.06	100
Gem superphosphate	0.0	0.00	100
Nitram and Gem superphosphate	29.3	2.56	100

* average of 6 replicates of 100 seeds per pretreatment.

Observations were made of the microbial growth when washings were taken from the samples. The commercially pretreated, nitram pretreated and Gem pretreated washings produced microbial growth consistent with that found on previous commercially pretreated seed. The Garotta pretreated and combined nitram and Gem pretreated samples produced more prolific microbial growth.

Commercially pretreated seed, Nitram pretreated seed and Gem Superphosphate pretreated seed showed no obvious changes in physical appearance throughout the pretreatment time. However the seed in the Nitram and Gem combination pretreatment and 1.0g Garotta pretreatment darkened in colour and some split open during the warm period of incubation.

The pretreatments run using surface sterilised seed remained sterile for the entire 24 weeks. No physical changes to the seed were noted during any of the sterile seed pretreatments - seed remained light brown in colour and did not show any signs of splitting open.

Table 5.3 shows the germination percentages for the surface sterilised seed following the various pretreatments. It can clearly be seen that no germination was found in any of the pretreatments where surface sterilised *Rosa corymbifera* 'Laxa' seed was used. All raw data can be found in appendix 5.1.

Table 5.3 Percentage germination and viability of surface sterilised *Rosa corymbifera* 'Laxa' seed following various pretreatments.

	germination* (%)	SE	viability* (%)
Commercial	0.0	0.00	100
1.0g Garotta	0.0	0.00	100
Nitram	0.0	0.00	100
Gem superphosphate	0.0	0.00	100
Nitram and Gem superphosphate	0.0	0.00	100

* average of 6 replicates of 100 seeds per pretreatment.

Table 5.4 shows the results for the pretreatments when Garotta was added weekly and also when the pretreatment incubation was reduced from 12 weeks in the warm to 6 weeks in the warm.

Table 5.4 Percentage germination of *Rosa corymbifera* 'Laxa' seed following a 6 week warm incubation (the usual 12 week incubation was conducted for comparison) as well as the addition of Garotta at weekly intervals.

	germination* (%)	SE
Commercial	3.5	0.80
1.0g Garotta	79.8	1.69
1.0g Garotta (6 week warm)	79.3	3.85
Weekly Garotta (6 week warm)	82.4	1.44

The results show quite clearly that the reduction of the warm incubation time of the pretreatment of *Rosa corymbifera* 'Laxa' with Garotta had no effect on germination percentage. The addition of Garotta on a weekly basis during the warm period of incubation also had no detrimental effect of germination. All raw data can be found in appendix 5.1.

5.1.1.3 Discussion

There is a very marked increase in percentage germination of *Rosa corymbifera* 'Laxa' following addition of Garotta when compared to the usual commercial method of pretreatment. The actual presence of Garotta was the overwhelming factor in enhancing germination percentage, not the amount added.

The difference between percentage germination obtained from the commercial pretreatment and both of the Garotta pretreatments was very highly significant. However, the difference between the 0.5g Garotta and 1.0g Garotta pretreatments was only significant at the 5% level. The amount of Garotta added would therefore appear to have only a slight effect on enhancement of germination.

It can be concluded that Garotta greatly enhanced germination of *Rosa corymbifera* 'Laxa' in the laboratory trials.

The germinations achieved with the Garotta pretreatment and commercial pretreatment were 95% and 2% respectively. If a nitrogen source alone or a phosphate source alone was capable of enhancing germination of *Rosa corymbifera* 'Laxa' following pretreatment, it would be expected that percentage germination would exceed that of the commercial pretreatment and be close to that of the Garotta pretreatment. However, the nitrogen source only reached 4% germination and the phosphate 0%. Therefore on their own these two substrates cannot enhance germination.

A combination of a nitrogen and phosphate source enhanced germination of *Rosa corymbifera* 'Laxa' to 29% (compared with 2% in the commercial pretreatment). It would appear that the combination does fulfill some of the requirements that Garotta provides. However the percentage germination achieved falls way short of that of the Garotta pretreatment. This could well be due to other important nutrients being absent.

As previously discussed, the growth of a microbial population is dependant upon the nutrient status of its immediate surroundings (Schlegel, 1993). The C/N ratio is of particular importance, as when this ratio is high, nitrogen is a limiting factor. Phosphorus has also been proposed as a limiting nutrient for microbial growth (Campbell, 1985).

Studying the results obtained here it would be logical to assume that whilst the combination of the nitrogen and phosphorus source provided some nutrients for microbial growth, another nutrient or factor was missing. Nitrogen and phosphorus were added at the same rate as contained in the equivalent of the 1.0g Garotta pretreatment. The difference in germination percentage between the two pretreatments was 66%. Thus other nutrients as well as these two are required to substitute for Garotta.

No germination was found with any of the pretreatments of *Rosa corymbifera* 'Laxa' when the seed had been surface sterilised. No microbial growth or activity was detected in any of the pretreatments for the duration of the incubation time. To ensure that the sterilisation procedure had not killed the embryo, viability was assessed and 100% of seed in all pretreatments was found to be viable. The mechanism by which the commercial pretreatment overcomes dormancy and the mechanism by which Garotta enhances this process appears to have been eliminated by surface sterilising the seed.

All other factors for the pretreatments of the sterilised seed were identical to those of the unsterilised seed described in the previous section (5.3). Yet the commercial pretreatment gave a germination of 2% and the 1.0g Garotta pretreatment resulted in a germination of 95%. Following surface sterilisation seed from the same seed batch gave 0% germination for both pretreatments.

This would give the clear conclusion that the microbial loading on *Rosa corymbifera* 'Laxa' and the subsequent growth of the microorganisms during the pretreatment enable the seed to germinate. This contrasts with the current practice whereby a grower may introduce fungicides to combat potentially harmful microbes (Morpeth *et al*, 1997), which in this system would result in zero germination.

It was also found that reducing the warm period of incubation to 6 weeks instead of the usual 12 weeks had no detrimental effect of percentage germination. This being the case the total pretreatment time can be reduced by one quarter to 18 weeks instead of the previously cited 24 weeks. This could have very important implications in the nursery business where time and resources are stretched during the harvest and pretreatment times of the year.

The addition of Garotta on a weekly basis did not have much effect on germination compared to the 1.0g Garotta pretreatment. This pretreatment effectively had six times the amount of Garotta added than the 'normal' 1.0g Garotta pretreatment during the warm period of incubation. There was only an increase of 3% in germination. It can be concluded that the nutrients in the 1.0g Garotta pretreatment are not limiting as the addition of extra Garotta does not enhance germination.

5.1.2 Germination trials of the other five native species

Laboratory and field trials were carried out on five other species as well as *Rosa corymbifera* 'Laxa', as discussed in the introduction to section 5. These are commonly grown native tree species produced under similar conditions to *Rosa corymbifera* 'Laxa'.

Each species has a commercially recommended pretreatment of warm and cold incubation for overcoming dormancy (Gordon & Rowe, 1982), summarised below in table 5.5. However, the comparative nature of this study meant that the five species listed in table 5.5 were given the same pretreatment conditions as *Rosa corymbifera* 'Laxa' (i.e. 12 weeks at 25°C followed by 12 weeks at 4°C).

Table 5.5 The recommended pretreatments for the five other native tree species trialled (information taken from Gordon & Rowe, (1982)).

Species	Duration of Pretreatment (weeks) Warm : Cold		Pretreatment medium
Acer campestre	4	12 - 24	compost*
Crataegus monogyna	4 - 8	12 - 16	compost*
Prunus spinosa	2	18	compost*
Rosa canina	8	8 - 12	compost*
Tilia platyphyllos	4 - 20	20 - 24	compost*

*compost refers to seived peat and moist sand

Objective; to test whether the addition of Garotta to the pretreatment regime applied to *Rosa corymbifera* 'Laxa' has a similar effect on other native tree seeds.

5.1.2.1 Materials and Methods

The pretreatment which has already been reported for *Rosa corymbifera* 'Laxa' (see section 2.3) was adopted for all species of seed. The same proportions of soaked seed to moist vermiculite were used (10g soaked seed to 25g moist vermiculite). The pretreatments used were the commercial pretreatment, 0.5g Garotta pretreatment and the 1.0g Garotta pretreatment. Again, six replicates were set up for each pretreatment of each species and if no problems were encountered during the incubation period with any of the replicates, 4 were selected at random for the trials.

Laboratory germinations were carried out on moist filter paper for *Rosa canina* and moist sand for the larger seeds of *Acer campestre, Crataegus monogyna, Prunus spinosa* and *Tilia platyphyllos* according to ISTA (1993). Four lots of 100 seeds were used from each of the 4 replicates, i.e. 1600 seeds per pretreatment per species.

5.1.2.2 Results

The laboratory germination results are shown in table 5.6. Each germination percentage represents the average of 4 lots of 100 seeds from each of 4 replicates per pretreatment (i.e. the average of 1600 seeds).

The addition of Garotta to the pretreatment of *Crataegus monogyna*, *Prunus spinosa* and *Rosa canina* enhanced the germination of these species when compared to the commercial pretreatment. The exceptions to this were *Acer campestre* and *Tilia platyphyllos* where no or extremely low germination was encountered in the laboratory trials.

Table 5.6 Laboratory germination results for the five native tree species.

	commercial		0.5g Garotta		1.0g Garotta	
	% germination	SE	% germination	SE	% germination	SE
Acer campestre	0.0	0.00	0.0	0.00	0.0	0.00
Crataegus monogyna	5.0	0.26	6.0	0.45	11.1	0.46
Prunus spinosa	4.8	0.36	8.6	0.36	10.1	0.42
Rosa canina	9.0	0.61	43.0	1.19	32.8	0.62
Tilia platyphyllos	0.6	0.15	0.0	0.00	0.8	0.25

There was a significant difference in germination between all pretreatments for all species, except with *Acer campestre* where no seeds germinated in any pretreatment. There was also no significant difference between the commercial pretreatment and 1.0g Garotta pretreatment for *Tilia platyphyllos*. Table 5.7 summarises the significance found between pretreatments for each species.

Table 5.7 The levels of significance between pretreatments for the laboratory germinations of each species of native tree (raw data can be found in appendix 5.2).

	commercial and 0.5g 'Garotta	commercial and 1.0g Garotta	0.5g Garotta and 1.0g Garotta
Acer campestre	n/a	n/a	n/a
Crataegus monogyna	1%	0.1%	0.1%
Prunus spinosa	0.1%	0.1%	5%
Rosa canina	0.1%	0.1%	0.1%
Tilia platyphyllos	0.1%	ns	1%

There was no significant difference between germinations of any replicates of any pretreatments in any species except the 1.0g Garotta pretreatment for *Prunus spinosa*.

5.1.2.3 Discussion

The germination percentages were very low for all species in the laboratory trials, except for *Rosa canina*, a very similar species to *Rosa corymbifera* 'Laxa'. However, in all cases there was significant improvement on germination with the Garotta pretreatments when compared to the commercial pretreatment (with the exception of the *Tilia platyphyllos* between the commercial and 1.0g Garotta pretreatments). There was also always significant difference between the 0.5g Garotta and 1.0g Garotta pretreatments.

Acer campestre showed no germination in the laboratory, therefore germination figures were not comparable from a statisitcal angle.

5.2 Field germination trials

The same seed batches which were used for the laboratory trials (5.1) were also used for the field trials so that a direct comparison could be made of the percentage germination. Six different native species (including *Rosa corymbifera* 'Laxa') were given the same three pretreatments as previously described (commercial, 0.5g Garotta and 1.0g Garotta).

Objectives - to show that after pretreatment with Garotta, percentage germination of *Rosa corymbifera* 'Laxa' was enhanced over the commercial pretreatment in the field. To determine whether the laboratory and field germination percentages were comparable and to report whether the laboratory system was an acceptable and valid alternative to the field trial.

The field trial was carried out at Oakover Nurseries, Ashford, Kent. The same methodology was used for the *Rosa corymbifera* 'Laxa' seed and all other species.The nursery was chosen as the trial could fit into an existing professional and commercially managed tree raising system. To conform to the ISTA guidelines (1993), 400 seeds were used from each replicate of each pretreatment. These were sampled as eight lots of 50 seeds, as the standard row width of the seed beds would not accommodate 100 seeds. The trial used two parallel seed beds with a row spacing of 10cm. The full design of the trial can be seen in appendix 5.3.

Seed was counted out into lots of 50 seeds and the bags individually labelled. These were kept cool and moist at all times by using sealed bags in cool boxes during transportation to the trial site. The six species were sown next to each other along the seed beds. The same design layout (see appendix 5.3) was used for all six species, the order of which was; *Crataegus monogyna, Rosa corymbifera* 'Laxa', *Prunus spinosa, Tilia platyphyllos, Rosa canina* and *Acer campestre.*

5.2.1 *Rosa corymbifera* 'Laxa' field trials

The field trial which included *Rosa corymbifera* 'Laxa' seed used the numbers of seed and replicates laid down by the ISTA guidelines (1993). A total of 4800 seeds were sown for this species, i.e. 1600 seeds per pretreatment.

5.2.1.1 Materials and Methods

Seed for the field trials was pretreated in the same manner as for the laboratory trials (section 5.1.1.1) and the same number of replicates used. Eight lots of 50 seed were counted from each replicate of each pretreatment and sown in rows in the field trial. The replicates were placed in a randomised manner according to the layout in appendix 5.3.

5.2.1.2 Results

The field germination percentages are shown in table 5.8. There is a very obvious enhancement of germination percentage in both pretreatments where Garotta was added (74% and 81%) when compared to the commercial pretreatment (21%). There was also a greater enhancement in the 1.0g Garotta pretreatment (81%) compared to the 0.5g Garotta pretreatment (74%).

Table 5.8 Percentage germination for the field trials on *Rosa corymbifera* 'Laxa' after 21 days. Raw data can be found in appendix 5.4.

	percentage germination*	SE
commercial	20.8	1.02
0.5g Garotta	74.1	1.94
1.0g Garotta	80.6	1.83

* average of 32 replicates of 50 seeds per pretreatment (i.e. 1600 seeds per pretreatment).

Statistically there was a very highly significant difference between the germination percentages of the commercial pretreatment and the 0.5g Garotta pretreatment, and also between the commercial pretreatment and 1.0g Garotta pretreatment. There was only significant difference at the 5% level between the 0.5g Garotta pretreatment and 1.0g Garotta pretreatment.

5.2.1.3 Discussion

The results show that when Garotta was added to the pretreatment of *Rosa corymbifera* 'Laxa' seed it greatly enhanced germination in the field compared with the commercial pretreatment. A four fold increase was recorded between the commercial pretreatment (21%) and 1.0g Garotta pretreatment (81%).

Whilst the addition of Garotta greatly enhanced germination percentage compared to the commercial pretreatment, the difference between germination percentages of the 0.5g and 1.0g rates was not nearly so marked.

These results follow the same trend as those for the laboratory trials. The commercially pretreated seed gave low percentage germination whilst both the Garotta pretreatments gave a very high percentage germination. The 1.0g Garotta pretreatment always showed enhanced germination over the 0.5g Garotta pretreatment.

5.2.2 Field trials of other native species

The field trial for the other five native species was conducted at the same time and place as for *Rosa corymbifera* 'Laxa', under identical conditions as described in sections 5.2 and 5.2.1.1.

5.2.2.1 Materials and Methods

Field germinations were conducted as described for *Rosa corymbifera* 'Laxa' in section 5.2.1.1. A total of 1600 seeds from each pretreatment for each species was used, a total of 4800 seeds per species.

5.2.2.2 Results

The germinations recorded in the field trials differed considerably from those in the laboratory. Table 5.9 shows the results for the field germinations. The addition of Garotta to the pretreatment of *Crataegus monogyna*, *Prunus spinosa and Rosa canina* enhanced germination when compared to the commercial pretreatment. This was particularly evident for *Rosa canina*. There was a slight reduction in germination with the addition of Garotta to *Tilia platyphyllos*, and no germination occurred in any pretreatments of *Acer campestre.*

Table 5.9 Field germination results for the five native tree species. Raw data can be found in appendix 5.5.

	commercial		0.5g Garotta		1.0g Garotta	
	% germination	SE	% germination	SE	% germination	SE
Acer campestre	0.0	0.00	0.0	0.00	0.0	0.00
Crataegus monogyna	47.9	7.46	53.6	7.77	55.0	6.93
Prunus spinosa	37.1	5.70	48.6	6.59	46.7	7.16
Rosa canina	17.9	8.94	62.8	10.93	64.5	13.52
Tilia platyphyllos	11.4	6.27	10.3	3.60	10.1	4.40

Statistical differences were found between the germinations of the commercial pretreatment and 0.5g Garotta pretreatment, and the commercial pretreatment and 1.0g Garotta pretreatments for *Crataegus monogyna*, *Prunus spinosa and Rosa canina*. No signicance was found between any of the Garotta pretreatments for any species. There was also no significance between the germination percentages recorded for the *Tilia*

platyphyllos pretreatments. *Acer campestre* did not germinate at all. This information is summarised in table 5.10.

Table 5.10 The levels of significance between pretreatments for the field germinations of each species of native tree (raw data can be found in appendix 5.5).

	commercial and 0.5g 'Garotta	commercial and 1.0g Garotta	0.5g Garotta and 1.0g Garotta
Acer campestre	n/a	n/a	n/a
Crataegus monogyna	1%	1%	ns
Prunus spinosa	1%	1%	ns
Rosa canina	0.1%	0.1%	ns
Tilia platyphyllos	ns	ns	ns

ns = no significance.
n/a = not applicable as no germination was recorded for any pretreatment.

No statistical differences were found between replicates for any pretreatment of any species with the exception of the commercial pretreatment for *Rosa canina* and *Prunus spinosa* and the 0.5g Garotta pretreatment of *Rosa canina*.

5.2.2.3 Discussion

The field trial results showed that the addition of Garotta to the pretreatment significantly enhanced germination *of Crataegus monogyna*, *Prunus spinosa* and *Rosa canina*. With these three species there was significant difference between the commercial and both Garotta pretreatments, however there was no significant differences between the two Garotta pretreatments. *Tilia platyphyllos* showed neither a significant enhancement or reduction in germination, whilst *Acer campestre* failed to germinate in any of the pretreatments.

The addition of Garotta to the other three native *Rosaceous* species (*Crataegus monogyna*, *Prunus spinosa* and *Rosa canina*) of seed has the same enhancing effect on germination as it does with *Rosa corymbifera* 'Laxa'.

5.3 Summary Discussion

In both the laboratory and field germination trials percentage germination was enhanced by the addition of Garotta to the pretreatment of the majority of species. The most marked effect was seen in *Rosa corymbifera* 'Laxa' and *Rosa canina*, although significant differences were found with *Crataegus monogyna* and *Prunus spinosa*.

Whilst the same trends in germination were found within the species both in the laboratory and the field, the field germinations were superior in all cases except the *Rosa corymbifera* 'Laxa'. It would therefore appear that the laboratory germination of *Rosa corymbifera* 'Laxa' is a fair indicator of field performance, but the same cannot be said for the other species.

Lower percentage germinations were obtained with the tree species other than *Rosa corymbifera* 'Laxa' probably due to the different time requirements for pretreatment. Table 5.5 listed the recommended pretreatment times for each of the species tested, none of which were the same. It is likely that if each species were given its own particular time requirements at the different temperatures, then percentage germination would have been higher. The fact that germination, particularly in the field, was respectable on a pretreatment based on the conditions for *Rosa corymbifera* 'Laxa' bodes well for further enhancement.

Addition of nitrogen or phosphate individually to the pretreatment of *Rosa corymbifera* 'Laxa' resulted in no enhancement of germination over the commercial pretreatment. A combination of the two did increase the germination percentage over the commercial pretreatment but to nowhere near the extent of Garotta.

Surface sterilising *Rosa corymbifera* 'Laxa' seed removed all microbes from the seed coat. Thus the pretreatments run with this seed had no microbial loading at the set up of the experiments. After completion of the warm and cold periods of incubation, no colour changes had occurred and no seed had split. Germination was zero in all pretreatments. The only factor missing was microbes, and thus they are essential for overcoming dormancy in this system. The seed was 100% viable at the end of the pretreatments, so damage during surface sterilisation could be ruled out.

6. DISCUSSION AND CONCLUSIONS

Under commercial pretreatment conditions low germination percentage was achieved with *Rosa corymbifera* 'Laxa'. Over the five year study period the percentage of seed germinated ranged from 2% to 63% (figure 2.1) with an average germination rate of 26%. Whilst the highest germination under commercial conditions of 63% would be acceptable to a grower, the lower figures, and indeed the average, would not. The extra volume of seed collected or purchased, and consequently the extra labour and land required to process such a species through pretreatment to sowing, would result in a very expensive crop of rootstocks.

The commercial conditions adopted were taken from standard nursery practices, using moist vermiculite as the pretreatment media. Alternatives such as peat and sand were not used due to the organic content which would potentially interfere with the aims and objectives of the studies undertaken in this thesis.

The addition of Garotta to the commercial pretreatment of *Rosa corymbifera* 'Laxa' enhanced germination to between 75% and 99% for the same five year period (figure 2.5). The average germination during this time was 89%. These results not only far exceed the commercial germination figures, but are also greater than those expected with acid scarification (Roberts, 1979), advocated by some as the most effective method for overcoming the hard seed coat imposed dormancy (Blundell & Jackson, 1971; Todd-Bockarie *et al*, 1993).

Thus germination was not only vastly increased in percentage terms, it was made more reliable in terms of expected germination. A range of only 24% was found between the highest and lowest germination of *Rosa corymbifera* 'Laxa' for the Garotta pretreatment, compared with a 61% spread for the commercial pretreatment. Therefore the addition of only 1g of Garotta per 10g moist seed resulted in a vast increase in percentage germination of *Rosa corymbifera* 'Laxa' compared to the standard commercial pretreatment. A much higher, predictable and reliable germination system was achieved.

These results were consistently produced during a 24 week period. This timescale fits neatly between seed harvest in the autumn and sowing in the spring. Some producers, including some Dutch Growers (J. D. Beeston, pers. comm.), still use the 'two year seed'

concept of Crocker (1948), whereby seed is not sown until two springs after harvest. This requires double the resources of the Garotta pretreatment, as for part of the year there would be two seasons' seed crops undergoing pretreatment. This is both extravagant and unnecessary, as the findings of this study show. In fact, not only can the pretreatment process be conducted in 24 weeks (12 weeks warm and 12 weeks cold), but overall germination percentages are increased over the commercial pretreatment with the addition of Garotta. Recent experiments also showed that the warm period could in fact be reduced to 6 weeks from 12 weeks with no loss in germination performance (table 5.4). This would give the grower the advantage of more time to process the seed, as the majority of native woody species are harvested during the autumn, and must be quickly processed into pretreatments to be able to fit in the 24 week period between harvest and spring sowing (Gordon & Rowe, 1982). Using the Garotta pretreatment not only enhances the percentage germination, it gives the grower more time in the extremely busy period of harvest to pretreat seed. This extends the time window during which the seed can be put into pretreatment ready for spring sowing, alleviating some of the pressures experienced by the growers (authors observations).

During the warm period of pretreatment *Rosa corymbifera* 'Laxa' seed turned dark brown in the presence of Garotta, but remained light brown (the colour of the seed at setup) in the commercial pretreatment (plates 3.22, 3.23 and 3.24). When investigated closely this colour change occurred within the first four weeks of pretreatment. Coinciding with this colour change was the splitting of over 95% of the Garotta pretreated seeds, but less than 5% in the commercial pretreatments. These criteria of colour change and splitting were soon found to be indicative of subsequent germination of those seeds.

These physical changes were relatively simple to detect and thus the 6 week warm period of pretreatment was tried based on these results. Germination percentage was not reduced after 6 weeks warm pretreatment with Garotta when compared to the 12 week pretreatment. Initial thoughts were that a composting effect was occurring to cause the colour change and splitting of *Rosa corymbifera* 'Laxa' seed in the presence of Garotta. Studies monitoring temperature and pH during the warm period of pretreatment showed no change in temperature when compared to ambient, or any changes in pH compared to a moist vermiculite, no seed, control (results in appendix 2.1). Therefore physical effects of microbial composting in the traditional sense were not found to be effective. Something more subtle was occurring during pretreatment.

The presence of microbes in the pretreatments was confirmed after testing the various components of the pretreatments for fungi and bacteria. The air dried seed used in the experiments was found to have a microbial loading on it (figure 2.3), whilst all the other components, vermiculite, sterile tap water and Garotta, did not. Further investigations were made from harvesting of the hips to the air dried seed. The seed inside the hips was found to be sterile (figure 2.4), however the outer surface of the hips on the bushes were loaded with fungi and bacteria. This initial inoculum was greatly increased during the extraction process which basically consisted of softening the flesh of the hips by fermentation. Washing of the seed reduced the inoculum level, however some remained on the seed of *Rosa corymbifera* 'Laxa'. The microbes found in the pretreatments of *Rosa corymbifera* 'Laxa' entered the pretreat mix on the seed. The seed became loaded with the inoculum whilst it was extracted from the hips, the outer surface of the hips being the initial carrier of the microbes.

Microbial numbers increased dramatically during the pretreatment of *Rosa corymbifera* 'Laxa' both in the commercial and Garotta pretreatments. Microbial numbers were always much greater in the presence of Garotta than in the commercial pretreatment. In some experiments a pretreatment was used with less Garotta than the 1.0g usually used. Microbial numbers were lower in these pretreatments than the 1.0g Garotta pretreatment, but higher than the commercial pretreatment (figures 4.1 and 4.2).

After 3 weeks both bacterial and fungal numbers decline (figures 4.1 - 4.4), probably due to a limiting factor. It is possible that the limiting factor was nitrogen. At the beginning of the warm period the C/N ratio would be favourable to microbial growth in the presence of the Garotta. The amount of nitrogen in the system was calculated to be far in excess of that recommended for laboratory culture media (Madigan *et al*, 1997). As this nitrogen was 'used up', the numbers of microbes peaked, then declined. However, large numbers of microbes were still present for the duration of the pretreatment. The likely explanation for this would be the succession of different species of microbes, each favouring different conditions. Garrett (1981) describes the general trend of fungal succession on a substrate. His work showed that as the substrate is colonised by different fungi and is broken down, other fungi are capable of growth. The effect of soluble soil nitrogen on saprophytic survival was also discussed, finding that 0.33g/litre ammonium nitrate gave maximum longevity of *Gaeumannomyces graminis* on wheat straw. Lower levels resulted in the fungus dying out due to carbohydrate starvation, higher levels exhausted the cellulose

prematurely. This level is remarkably similar to that encountered in Garotta, and following the theme of Garrett, nitrogen is likely to become limiting within a short time - as found in the experiments conducted for this thesis. It would therefore appear that whilst the initial level of nitrogen supplied by the ammonium salts in Garotta was sufficient for rapid microbial growth over the first three weeks, it soon became limiting and numbers declined. However, recycling of that nitrogen may explain the continued survival of some fungi and bacteria, as could availability of soluble carbon sources released by the primary decomposers which initially break down the cellulose (Lacey, 1988). How succession develops is determined by how the nutrient status changes with time. It is also evident that whilst the microbial numbers declined in subsequent weeks, the effect on the *Rosa corymbifera* 'Laxa' seed had occurred (reflected in the colour change and splitting of the seed coat). This resulted in the enhanced germination.

The change in colour of the seed coat of *Rosa corymbifera* 'Laxa' pretreated with Garotta was most likely to have been caused by fungi. Many fungi are capable of degrading different forms of carbon sources, including some species which preferentially degrade cellulose and hemicellulose (Schlegel, 1993). This particular group of fungi are known as the 'brown rot' fungi due to the brown residues of phenyl propane polymers they leave. This would explain the darkening brown over the initial weeks of pretreatment. Decomposition of wood (i.e. the seed coat) is a very slow process (Schlegel, 1993) and this would explain why no obvious physical degradation was observed on these seeds.

Eventually the nitrogen source would have dwindled and run out, and this was reflected in the decline in microbial numbers after 4 weeks. The continued survival of the microbes may be due to 'recycling' of nitrogen from dead fungi and bacteria. Transferring the pretreatments into the cold (end of week 12) did not decrease the numbers of microbes present, only their potential for growth. The results in figures 4.3 and 4.4 showed that bacterial and fungal numbers were the same in the cold as they were in the warm. However whilst they were not capable of growth under the cold conditions (figures 4.3 and 4.4, time point 14a), subsequent incubation under favourable temperatures saw equal numbers of microbes to those when initially incubated under warm conditions (figures 4.3 and 4.4, time points 14 and 14b). A closer inspection of fungal numbers (figure 4.5) showed that the maximum number of fungal colonies was reached after 2 weeks, and subsequently numbers trailed off after 3 weeks.

The monitoring of microbial numbers gave a good indication that the addition of Garotta to the pretreatment of *Rosa corymbifera* 'Laxa' seed had a profound effect on the total numbers of microbes compared to the commercial pretreatment. Perhaps more relevant than these counts would be the activity of the microbes *in situ*. The microbial counts were measured as colony forming units, and many colonies could have originated from one hypha, for example. Therefore, whilst useful, the microbial counts were limited as an indicator as to the potential activity of the microbes within the pretreatment mix. Two further approaches were used to assess this activity - the cotton strip assay and the fluorescein diacetate (FDA) assay. The former was used to assess the population of microbes in any given week capable of cellulose degradation, the latter initially giving a quantitative assessment of the whole microbial population regardless of species, and then also as an assessment of the extracellular enzyme potential of the microbes. The FDA assay on the 1g samples estimated the microbial population at a specific time point, but when cotton squares were introduced the extracellular enzymes were assessed from a 7 day incubation.

What was found using these methods was that the microbial activity of the 1g samples followed a similar pattern to the microbial numbers. Higher activity was always found in the Garotta pretreatment compared to the commercial pretreatment (figure 4.8). There was no decline in activity once the pretreatments were placed in the cold. At first this was an unexpected result, as microbial activity was expected to be minimal at 4°C. However, the microbes do not die in the cold period of pretreatment, they remain live but dormant. Therefore when samples of the pretreatment mix were taken and assessed using the FDA assay, their potential activity was recorded. The true activity of the microbes would be very low at 4°C. However this particular technique would not be able to record real activity at low temperatures as samples are incubated under warm conditions (37°C for 30 minutes).

Microbial activity measured using the FDA assay on cotton squares gave a good indicator of actual enzyme activity. Cotton squares were placed into the pretreatments after 6 weeks to assess the extracellular activity of the microbes (figure 4.9). This was measured to give an assessment of the potential of the microbes to degrade a substrate. This was done by measuring the activity of enzymes produced by the microbes on cellulose (the cotton squares). The assessment on the 1g samples would measure both extra and intracellular activity, whilst the cotton squares would only measure the extracellular component. Placing cotton squares in the pretreatments as a carbon source for the microbes to attack

gave a representative assessment of microbial activity. Enzyme activity was significantly higher in the Garotta pretreatment than the commercial pretreatment. During the warm period of incubation there was a very large difference in activity between the commercial and Garotta pretreatments (figure 4.9). This indicates a high degree of extracellular excretion of enzymes to break down the cotton. The much higher activity in the presence of Garotta was most likely due to the more favourable (lower) C/N ratio caused by the addition of the Garotta. This gives much more favourable conditions for microbial growth (Campbell, 1985).

Once placed into the cold, activity in both the commercial and Garotta pretreatments fell to a very low level. This is consistent with the hypothesis that whilst the microbes remain viable they are no longer active in the cold. Thus no enzymes would be excreted and none would be detected on the cotton. This contrasts with the 1g samples which actually measured the enzymes contained within the viable but dormant microbes. These results showed that the activity measured by the FDA assay came from the microbes, either as total enzyme values (intra and extracellular) as measured by the 1g sampling, or as extracellular values as measured by the cotton squares. Activity was always greater in the presence of Garotta, showing that microbial activity at the enzymatic level is stimulated by Garotta due to increasing microbial numbers. Thus, the more microbes the higher the total activity.

Measuring extracellular microbial activity on cotton squares showed elevated levels of enzyme activity in the Garotta pretreatment. This method would also measure any other enzymes excreted by the microbes. Therefore a method was used which indirectly measured the population of microbes capable of degrading cellulose in the different pretreatments. This was the cotton strip assay, which depended upon the weakening of cotton by cellulytic enzymes produced by the microbes (section 4.3).

During commercial pretreatment of *Rosa corymbifera* 'Laxa', little tensile strength was lost from the cotton strips, indicating a low population of microbes capable of cellulase production. These results are consistent with the low numbers counted for this pretreatment as well as the low activity found with the FDA assay. A lower level of tensile strength loss than the commercial pretreatment was found in the absence of seed (figure 4.10). This may be due to the presence of the seed in the commercial pretreatment acting as a consistent nutrient source, whereas in the absence of the seed (vermiculite only), the cotton strips are

the only source of carbon. This is confirmed by the results of the tensile strength loss being similar for both the vermiculite only and commercial pretreatments for the first 3 weeks, but a higher loss in subsequent weeks in the commercial pretreatment. The microbes will probably have become established in higher numbers throughout the pretreatment mix in the presence of seed, and not in the absence of seed.

The Garotta pretreatment (with seed) showed high levels of tensile strength loss due to the elevation of microbial numbers from the initial inoculum on the seed. The high level of cellulase activity would be promoted by the relatively high level of nutrients available to the microbes from the Garotta. These nutrients would give a more favourable C/N ratio to the microbes. The Garotta only pretreatment also showed high levels of tensile strength loss in the absence of the seed. These experiments were not setup sterile and hence this activity would probably have resulted from contaminant microbes. Without the 'distraction' of the seed and with the elevated nutrient source from the Garotta, the C/N ratio and hence microbial activity would favour the cotton strips as the carbon source. Hence a high level of tensile strength loss was found.

When all pretreatments were placed into the cold (figure 4.10), the tensile strength loss was minimal, indicating a sharp decline in cellulase activity. This confirms the findings of the FDA assay which showed that activity of microbes is dramatically reduced under cold conditions, although they still remain viable.

Tensile strength loss was always greatest in the Garotta pretreatment compared to the commercial pretreatment (figures 4.10, 4.11 and 4.12). Figure 4.10 also shows the effect of the half rate of Garotta on the tensile strength loss, and whilst the results were always higher than the commercial pretreatment, they did not reach the levels found in the Garotta pretreatment. This would suggest that the lower nutrient input of this pretreatment (compared to the 1.0g Garotta pretreatment) was sufficient to decrease the C/N ratio to more favourable levels for microorganisms than the commercial pretreatment. However this was not to the same extent as the 1.0g Garotta pretreatment.

The results obtained for the microbial counts, microbial activity and microbial population assessments were confirmed by the visual assessments of the pretreated seeds (section 4.4). Low levels of inoculum were found on the air dried seed prior to it being used in the

pretreatments. The potential microbial population was then realised by incubating this seed in a humid environment conducive to microbial growth.

Seed sampled during the course of the pretreatments was found to have higher levels of microbial growth across the seed coat surface comparable to the activity found with the studies on microbial numbers and activity. *Rosa corymbifera* 'Laxa' seed sampled later into the pretreatments (weeks 12 and 14) showed larger quantities of microbial structures, however close study of these plates indicates that much of the growth was old and probably no longer viable.

Consistently higher percentage germination was achieved with *Rosa corymbifera* 'Laxa' when Garotta was added to the pretreatment when compared to the commercial pretreatment. These findings were found in both the laboratory and field trials. Germination was lower in the 0.5g Garotta pretreatment compared to the 1.0g Garotta pretreatment (81% vs. 93% - table 5.1). Substituting Garotta with an equivalent amount of nitrogen or phosphorus had no effect on germination compared to the commercial pretreatment. However, when combined, a germination of 29% was obtained (compared to 2% in the commercial pretreatment). Hence, whilst these two components of Garotta enhance germination compared to the commercial pretreatment, other factors are missing or limiting.

Additions of Garotta on a weekly basis, whilst enhancing the nutrient status of the pretreatment, had no effect on germination compared to the Garotta pretreatment (table 5.4). When microbes were totally excluded from the pretreatments by surface sterilisation of the seed, subsequent germination was totally inhibited. Thus the microbial loading and growth are paramount to the enhanced germination found with the Garotta pretreatment. A total absence of microbes resulted in no germination in any pretreatment, yet even where low numbers were present (the commercial pretreatment), germination was low.

The key findings relating to the pretreatment of *Rosa corymbifera* 'Laxa' are shown in the summary flow diagram (figure 6.1). The salient points found during the microbial counts, microbial activity (FDA assay) and microbial population (cotton strip assay) are compared between the commercial and Garotta pretreatments. Each is shown at the relevant time point during the pretreatment.

When the pretreatments were used under identical conditions for five other native species, encouraging results were obtained. Germination was enhanced by the addition of Garotta in 3 of the species in the laboratory, with no detrimental effect on the other 2 species. This result was also obtained in the field trial, with an overall higher percentage germination obtained in all species which germinated. However, these trials were conducted for the same pretreatment requirements as for *Rosa corymbifera* 'Laxa'. No allowances were made for the differences recommended by Gordon and Rowe (1982) in the cold period of incubation. In all but one species, a longer cold pretreatment is recommended. It would therefore be useful to study the Garotta pretreatment and commercial pretreatment in light of this, compensating where necessary for the differences in incubation time.

CONTAINS PULLOUTS

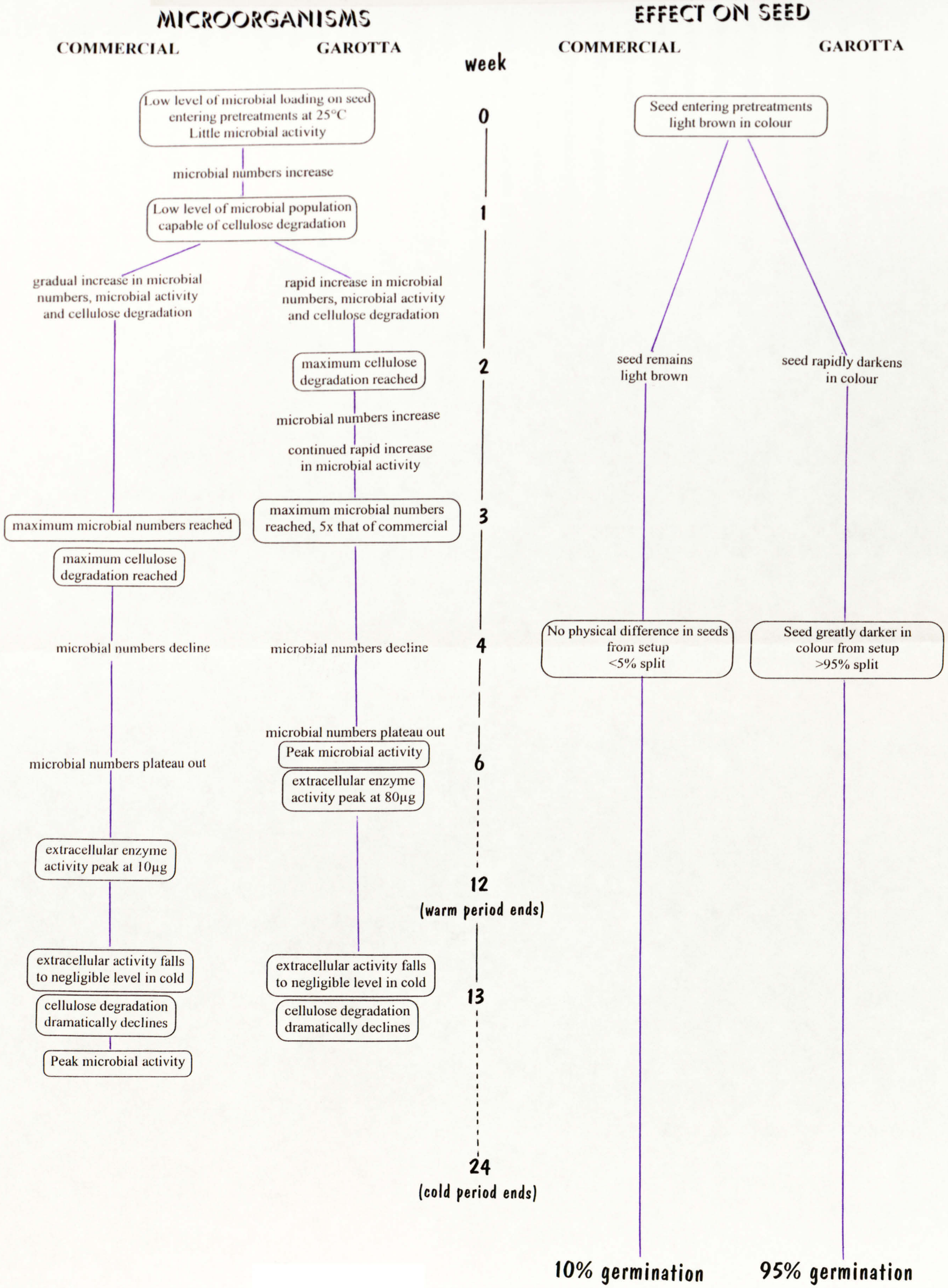


Figure 6.1 Summary diagram showing the key features found during the pretreatment of *Rosa corymbifera* 'Laxa'.

The cold period of the pretreatment (chilling) in moist conditions represents the environmental stimulus which promotes germination (Pinfield & Dungey, 1985). It is generally believed to be a fairly consistent time requirement within a species, however much variation is encountered between species (Gordon & Rowe, 1982; Pinfield & Dungey, 1985). It is this period of the pretreatment which overcomes the physiological dormancy described in section 1.2. Many studies have concentrated on the hormonal aspect of the chilling process to explain dormancy in woody species. Consensus agrees that abscisic acid (ABA) may contribute to either causing dormancy or preventing germination, whilst some other groups of growth regulators act as antagonists to ABA (Bewley & Black, 1994; Tillberg & Pinfield, 1982; Roberts & Hooley, 1988; Jackson, 1968). This dormancy can be described as either coat or embryo imposed, although ultimately it is the effect on the embryo which prevents or allows germination. Thus, hormonal inhibitors to germination are located in the embryo, seed coat, or both. Tillberg (1983) confirmed the findings of Jackson and Blundell (1963) that seeds of *Rosa* have a coat imposed dormancy. Both found that endogenous ABA decreased during a cold treatment. Yambe and co-workers (1992) conducted germination tests on the whole seeds, embryos only and embryos and seed coats together of *Rosa multiflora*. They found that intact seeds failed to germinate, whilst embryos on their own reached 75% germination. When embryos were incubated in the presence of excised seed coats, only 38% germination was recorded. These workers also found that leaching of ABA was more effective at 5°C than 25°C in water, and improved even more in the presence of activated charcoal. The same effect on germination (50%) after 25 weeks pretreatment at 5°C was obtained after only 3 weeks in cold water.

The debate as to the exact nature of physiological dormancy, a phenomenon manifested in tree seed, is unresolved. Growth regulators are implicated as the cause of dormancy (Lewark & Rudnicki, 1977; Bewley & Black, 1994), and specifically ABA would appear to have a hormonal block on germination (Roberts & Hooley, 1988). The cold period of pretreatment given to the *Rosa corymbifera* 'Laxa' seed would appear to have the effect of removing this type of dormancy. Both the commercial and Garotta pretreatment underwent 12 weeks at 4°C which mimics this cold period which would normally be the winter months shortly after harvest. This shows that under entirely natural conditions the seed could not be subjected to a sufficient warm period prior to the first winter after harvest, hence the two year requirement stated by Crocker (1948) and still used by some nurserymen today.

OVERALL CONCLUSIONS

- The addition of 'Garotta', a compost activator, to the pretreatment *of Rosa corymbifera* 'Laxa' enhanced germination in the laboratory and the field significantly compared to the commercial pretreatment.

- The addition of Garotta to the pretreatment of *Rosa corymbifera* 'Laxa' greatly reduced variation in percentage germination between harvests of different years.

- The addition of 'Garotta' to the pretreatment mix of *Rosa corymbifera* 'Laxa' significantly increased microbial numbers, microbial population and microbial activity.

- The microbial loading entering the pretreatment mix originated from the *Rosa corymbifera* 'Laxa' seed. The seed became inoculated during extraction from the hips.

- The presence of the microbes during the pretreatment was essential for germination in the commercial pretreatment.

- The addition of fungicides to pretreatment of woody seeds may prove to be detrimental in terms of resulting germination percentage.

- When the microbes in the pretreatment were enhanced using 'Garotta', germination was greatly increased over the commercial pretreatment. The effect on the seeds was shown to occur within 4 weeks of the warm period.

- The shorter time required to pretreat seed with Garotta (18 weeks) compared to commercial pretreatment (>24 weeks) gives the grower much more time to process the annual seed crop.

- The presence and effect of the microbes is consistent, i.e. the same enhancement of germination is found each year.

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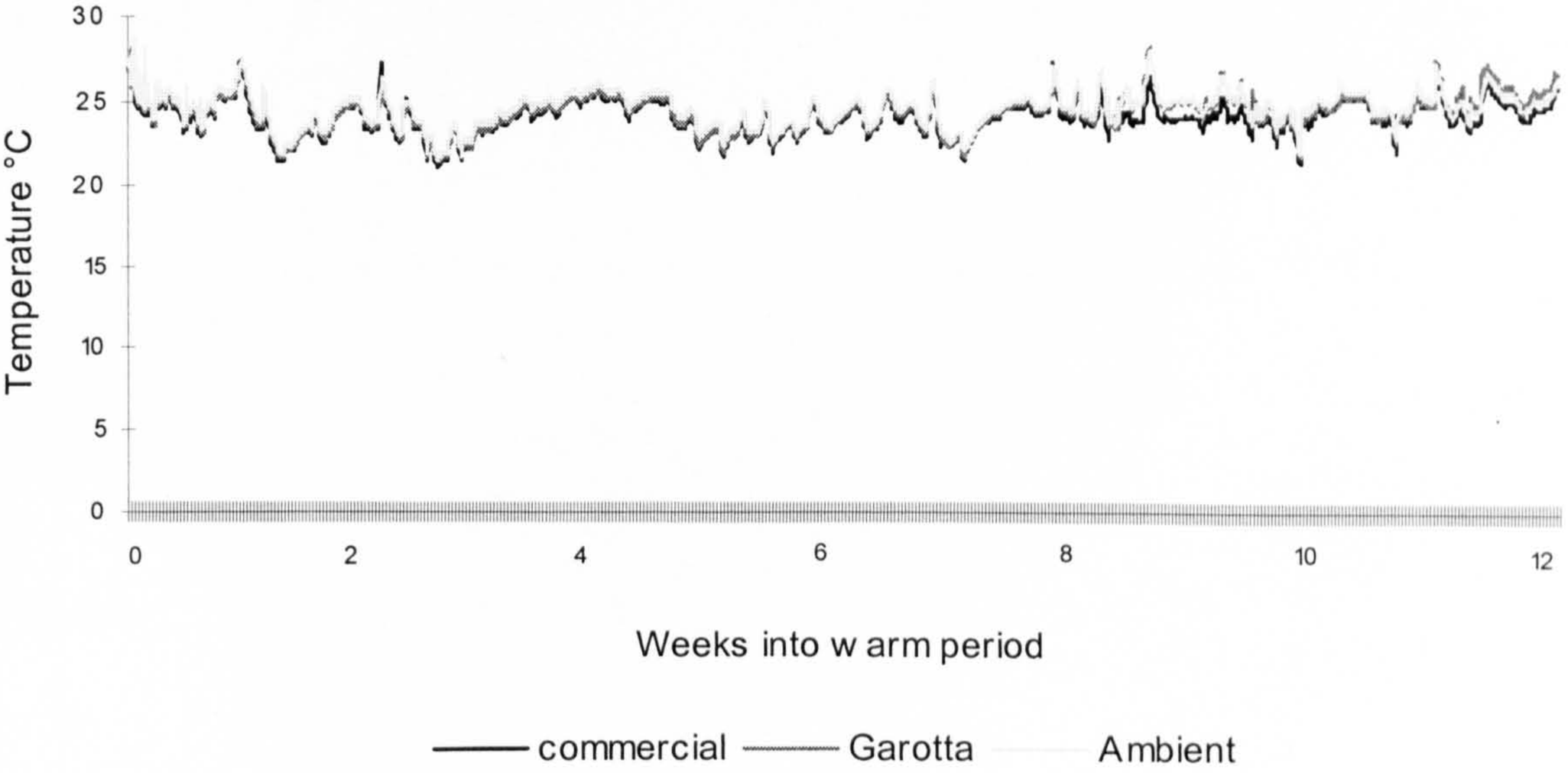
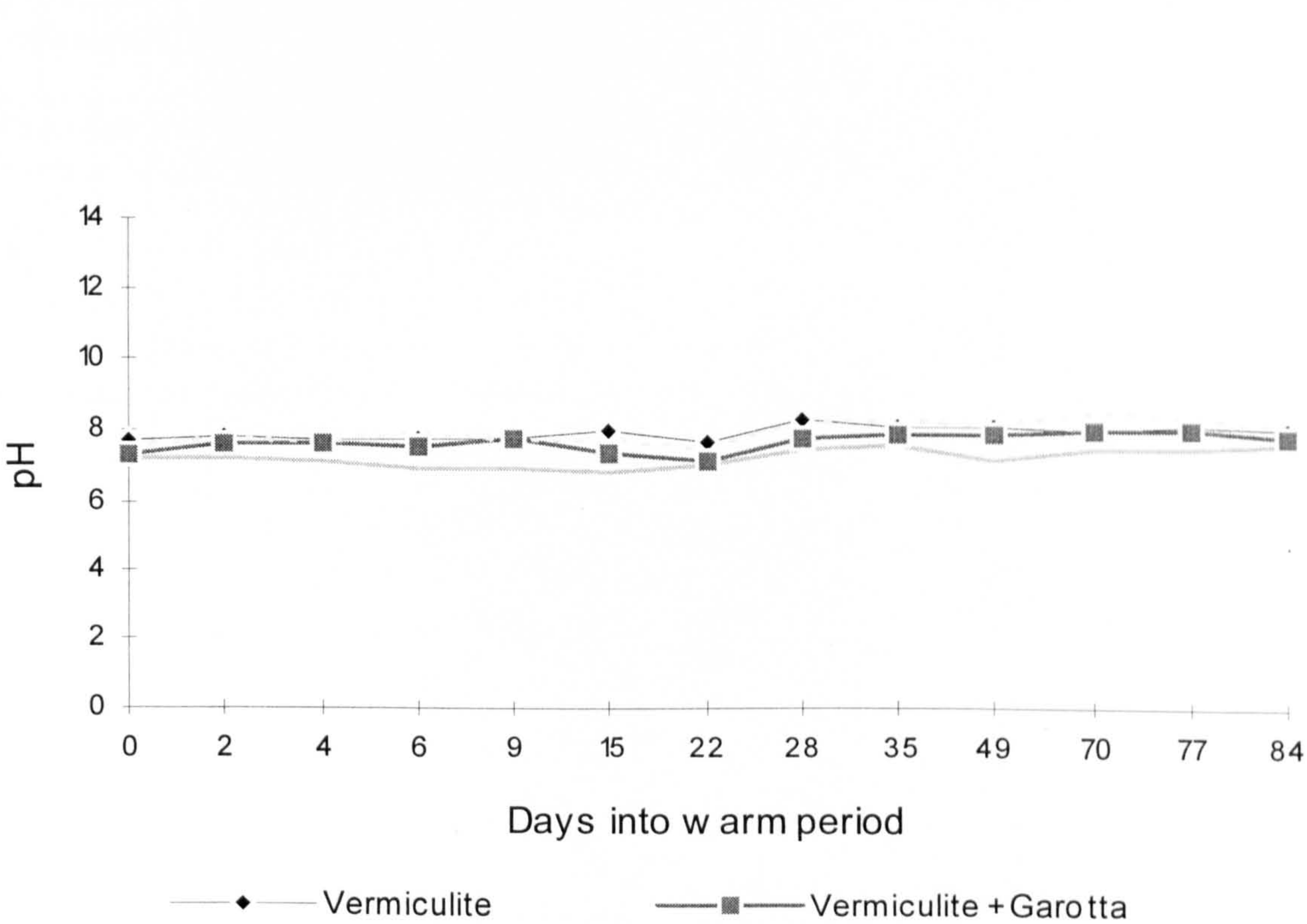
APPENDIX 1.1
Definitions of Terms

During the course of this thesis, certain words and terms are used which can have ambiguous use. Others have different definitions depending upon who is using them. The following commonly used terms are therefore defined in the context of this work.

- **Dormancy**:- the condition of a seed which does not germinate in otherwise conducive conditions (i.e. favourable moisture, oxygen and temperature) (Bradbeer, 1988).
- **Viable**:- a measure of the capacity of a seed or seed lot to germinate (U.S. Department of Agriculture, 1974). Viability is expressed as a percentage.
- **Germination**:- resumption of active growth in the embryo of a seed, demonstrated by the emergence of the axis, usually the radicle germinate (U.S. Department of Agriculture, 1974).
- **Stratification**:- the long standing practice used with tree and shrub species to overcome dormancy by chilling. Seed is placed in moist substrata and sometimes arranged in layers (hence 'stratified') for varying lengths of time depending upon species (Bewley & Black, 1994).
- **Pretreatment**:- during the course of this work this term will be used to describe a set of conditions the seeds were subjected to prior to germination tests. Stratification forms part of this pretreatment (authors interpretation).
- **Orthodox**:- the category of seeds which can be stored in a state of low moisture content for extended periods of time whilst remaining viable (Roberts, 1973). Many important species fall into this category including *Prunus* spp (cherry, blackthorn etc.), *Crataegus* spp (hawthorn etc.), *Acer* spp, *Rosa* spp, *Sorbus* spp and *Tilia* spp.
- **Recalcitrant**:- a smaller group of species whose seed must be stored at relatively high moisture contents to remain viable. However, their life span is short and at best may last a couple of months - this would not allow survival beyond one season (Roberts, 1973). Examples include many important broad-leaved tree species such as *Aesculus* spp, *Quercus* spp, *Corylus avellana*, *Fagus sylvatica* and some *Acer* spp.
- **Grading**:- the process by which tree seedlings which are transplanted or sold are divided by quality and age. Poor individuals would be disposed of (Mason, 1994).
- **Scarification**:- purposeful injury of the impermeable seed coat using either chemical or mechanical abrasion (Gordon & Rowe, 1982).
- **Chitting**:- emergence of the radicle from imbibed seed. Seed lots at this stage are ready for sowing (Gordon & Rowe, 1982).

APPENDIX 2.1
Temperature and pH during the warm period of pretreatment of *Rosa corymbifera* 'Laxa'





APPENDIX 3.1
Staining methods

1) Lignin

Materials
Phloroglucinol, 1% in 70% ethanol
Concentrated hydrochloric acid

Method
Flood sections for 5 minutes with phloroglucinol solution.
Pour off excess phloroglucinol and cover sections with a few drops of concentrated hydrochloric acid.

Result
Lignified tissue stains red.

2) Cellulose

Materials
Gram's iodine
75% sulphuric acid

Place sections in a drop of Gram's iodine. Examine for blue colouration.
Place a drop of the sulphuric acid onto the edge of the iodine and sections.

Results
Cellulose swells due to hydrolysis to hydrocellulose.
Other plant substances give a blue colour reaction with iodine, so locality must be checked prior to addition of acid. Any areas remaining blue and swelled were cellulose.

APPENDIX 3.2
Seed count - to determine average number per hip

Hip number	Number of seeds
1	20
2	20
3	19
4	18
5	20
6	20
7	19
8	19
9	19
10	18
11	19
12	19
13	18
14	18
15	19
16	19
17	18
18	17
19	19
20	19
Total	**377**
Average	**18.85**

APPENDIX 3.3

Split seed counts after the commercial (c) and Garotta (G) pretreatments

100 seeds per replicate, 4 replicates per pretreatment

	Replicate									Replicate								
	1		2		3		4			1		2		3		4		
	c	G	c	G	c	G	c	G		c	G	c	G	c	G	c	G	
1	×	✓	✓	✓	×	✓	×	✓	51	×	✓	×	✓	×	✓	×	✓	
2	×	✓	×	✓	×	✓	×	✓	52	×	✓	×	✓	×	✓	×	✓	
3	×	✓	×	✓	×	✓	×	✓	53	×	✓	×	✓	×	×	×	✓	
4	×	✓	×	✓	×	✓	×	✓	54	×	✓	×	✓	×	✓	×	✓	
5	×	✓	×	✓	×	✓	×	✓	55	×	✓	×	✓	✓	✓	×	✓	
6	×	✓	×	✓	✓	✓	×	✓	56	×	✓	×	✓	×	×	×	✓	
7	×	×	×	✓	×	×	×	✓	57	×	×	×	✓	×	✓	×	✓	
8	✓	✓	×	×	×	✓	×	✓	58	×	✓	×	✓	×	✓	×	✓	
9	×	✓	×	✓	×	✓	×	✓	59	×	✓	×	✓	×	✓	×	✓	
10	×	✓	×	✓	×	✓	×	✓	60	×	✓	×	✓	×	✓	×	✓	
11	×	✓	×	✓	×	×	×	×	61	×	✓	×	✓	×	✓	×	✓	
12	×	✓	✓	✓	×	✓	×	✓	62	✓	×	×	✓	✓	✓	×	✓	
13	✓	✓	×	✓	×	✓	×	✓	63	×	✓	×	✓	×	✓	×	✓	
14	×	✓	×	✓	×	✓	×	✓	64	×	✓	✓	✓	×	✓	×	✓	
15	×	×	×	✓	×	✓	×	✓	65	×	✓	×	✓	×	✓	×	✓	
16	×	✓	×	✓	×	✓	×	✓	66	×	✓	×	✓	×	✓	×	✓	
17	×	✓	×	✓	✓	✓	×	✓	67	×	✓	×	✓	×	✓	×	✓	
18	×	×	×	✓	×	✓	×	×	68	×	✓	×	✓	×	✓	×	✓	
19	×	✓	×	✓	×	✓	×	✓	69	×	✓	×	×	×	✓	×	✓	
20	×	✓	×	✓	×	✓	×	✓	70	×	✓	×	✓	×	✓	×	×	
21	×	✓	×	✓	×	✓	×	✓	71	×	✓	×	✓	×	✓	×	✓	
22	×	✓	×	✓	×	✓	×	✓	72	×	✓	×	✓	×	×	✓	×	
23	×	✓	×	✓	×	✓	×	✓	73	×	×	×	✓	×	×	×	✓	
24	×	✓	×	✓	×	×	×	✓	74	×	✓	×	✓	✓	✓	×	✓	
25	×	✓	×	✓	×	✓	×	✓	75	×	✓	×	×	×	✓	×	✓	
26	×	✓	×	✓	×	✓	×	×	76	×	✓	✓	✓	×	✓	×	✓	
27	×	✓	×	✓	×	✓	×	×	77	×	✓	×	✓	×	✓	×	✓	
28	×	✓	×	✓	×	✓	×	✓	78	×	✓	×	×	×	✓	×	✓	
29	×	✓	×	✓	×	✓	×	✓	79	×	✓	×	✓	×	✓	×	✓	
30	×	✓	×	×	×	✓	×	✓	80	×	✓	×	✓	×	✓	×	✓	
31	×	✓	×	✓	×	✓	✓	✓	81	×	✓	×	✓	×	✓	×	✓	
32	×	✓	×	✓	×	✓	×	✓	82	×	✓	×	✓	×	✓	×	✓	
33	×	×	×	✓	×	✓	×	✓	83	×	✓	×	✓	×	×	×	✓	
34	×	×	×	✓	×	✓	×	✓	84	×	✓	×	✓	×	✓	×	✓	
35	×	✓	×	✓	×	✓	×	✓	85	×	✓	×	✓	×	✓	×	✓	
36	×	✓	×	✓	×	✓	×	✓	86	×	✓	✓	✓	×	✓	×	×	
37	×	✓	×	✓	×	✓	×	✓	87	×	✓	×	✓	×	×	×	✓	
38	×	✓	×	✓	×	✓	×	✓	88	×	×	×	✓	×	✓	×	✓	
39	×	✓	×	✓	×	✓	×	✓	89	×	✓	×	✓	×	✓	×	✓	
40	×	✓	×	✓	×	✓	×	✓	90	×	✓	×	✓	×	✓	×	✓	
41	×	✓	×	✓	×	✓	×	✓	91	×	✓	×	✓	×	✓	×	✓	
42	×	✓	✓	✓	×	✓	✓	✓	92	×	✓	×	✓	×	✓	✓	✓	
43	×	✓	×	✓	×	✓	×	✓	93	×	✓	✓	×	×	✓	×	✓	
44	×	✓	×	✓	×	✓	×	✓	94	×	✓	×	✓	×	×	×	✓	
45	×	✓	×	×	×	×	×	✓	95	×	✓	×	✓	×	✓	×	✓	
46	×	✓	×	✓	×	✓	×	✓	96	×	✓	×	✓	×	✓	×	✓	
47	×	✓	×	✓	×	✓	×	✓	97	×	✓	×	✓	×	✓	×	✓	
48	×	×	×	✓	×	✓	×	✓	98	×	✓	×	✓	×	✓	×	✓	
49	×	✓	×	✓	×	✓	×	✓	99	×	✓	×	×	×	✓	×	✓	
50	×	✓	×	✓	×	✓	×	×	100	×	✓	×	✓	×	✓	×	×	

× = unsplit ✓ = split

Average Commercial = 4.75% split

Average Garotta = 91.5% split

APPENDIX 4.1
Bacterial and fungal numbers for the first 10 weeks of pretreatment

Bacterial numbers;

	1.0g 'Garotta'			0.5g 'Garotta'			commercial		
	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3
week									
0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
1	10500000	10500000	5900000	32000000	8500000	29000000	1300000	1880000	2300000
	9500000	8700000	8300000	27000000	12500000	19000000	1900000	1790000	1500000
	7700000	6400000	9300000	27000000	2300000	25000000	2190000	1450000	1900000
2	60000000	46000000	53000000	22000000	17000000	19000000	13400000	12000000	8000000
	74000000	49000000	52000000	27000000	18500000	19000000	12000000	8700000	8900000
	60000000	59000000	48000000	23000000	17300000	21000000	12600000	7700000	6400000
3	71000000	97000000	113000000	42300000	38000000	60000000	19000000	14200000	21000000
	76000000	99000000	66000000	41000000	50000000	44000000	17000000	11300000	31000000
	101000000	142000000	92000000	40500000	55000000	46000000	14000000	15500000	18000000
4	16000000	8000000	21000000	10800000	12000000	4100000	6800000	7000000	3900000
	15000000	14000000	11000000	10300000	13100000	5000000	8900000	5700000	4900000
	10000000	16000000	12000000	8200000	10900000	7100000	8500000	4000000	6200000
6	29800000	29000000	36000000	15200000	14500000	10900000	11800000	13600000	5400000
	30000000	32900000	37000000	26000000	14100000	13900000	8800000	13500000	4900000
	32300000	32100000	40000000	19500000	20600000	11900000	9800000	11200000	6100000
10	12600000	13800000	12900000	6700000	6200000	5800000	2050000	1600000	400000
	14000000	13900000	13900000	6750000	5700000	6000000	1770000	2800000	400000
	13300000	13850000	13400000	6600000	5950000	5900000	1910000	2200000	400000

Mean, Standard error and significant differences within pretreatments (i.e. between replicates) for bacterial numbers.

	Commercial			0.5g 'Garotta'			1.0g 'Garotta'		
	Mean	SE	significance	Mean	SE	significance	Mean	SE	significance
Week	($x10^8$)			($x10^8$)			($x10^8$)		
0	na	na	na	na	na	na	na	na	na
1	0.02	0.00	ns	0.05	0.00	ns	0.09	0.01	ns
2	0.10	0.01	ns	0.20	0.03	ns	0.56	0.04	ns
3	0.18	0.02	ns	0.80	0.09	ns	0.95	0.08	ns
4	0.06	0.01	ns	0.09	0.01	ns	0.14	0.01	ns
6	0.09	0.01	0.1%	0.16	0.02	ns	0.33	0.01	ns
10	0.02	0.00	1%	0.06	0.01	1%	0.14	0.00	ns

na = not applicable as no bacteria detected

Fungal numbers;

	1.0g 'Garotta'			0.5g 'Garotta'			commercial		
	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3
week									
0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
1	1800000	1200000	1400000	230000	340000	200000	128000	220000	200000
	1600000	1500000	1600000	270000	250000	500000	118000	160000	300000
	1400000	1400000	1400000	240000	340000	600000	116000	140000	180000
2	43000000	82000000	16000000	36000000	30000000	30000000	1200000	2600000	3000000
	28000000	50000000	36000000	34000000	30000000	20000000	3700000	2700000	2300000
	55000000	67000000	27000000	40000000	20000000	31000000	2300000	1700000	3400000
3	160000000	13000000	119000000	48000000	33000000	40000000	47000000	45000000	17000000
	180000000	117000000	102000000	49000000	37000000	37000000	35000000	31000000	22000000
	150000000	120000000	100000000	55000000	49000000	33000000	42000000	46000000	15000000
4	300000	300000	2000000	3600000	1600000	1200000	800000	600000	700000
	200000	200000	100000	1700000	900000	1100000	700000	600000	900000
	350000	100000	100000	120000	400000	1200000	700000	800000	900000
6	1400000	1500000	1200000	600000	1500000	900000	nd	nd	300000
	600000	1400000	1000000	800000	750000	1000000	10000	200000	300000
	600000	1200000	2000000	1300000	100000	900000	200000	nd	800000
10	400000	500000	800000	150000	900000	900000	800000	400000	100000
	800000	800000	1800000	100000	120000	1200000	1600000	1600000	300000
	600000	650000	1300000	125000	510000	1050000	1200000	1000000	200000

Mean, Standard error and significant differences within pretreatments (i.e. between replicates) for fungal numbers.

	Commercial			0.5g 'Garotta'			1.0g 'Garotta'		
	Mean	SE	significance	Mean	SE	significance	Mean	SE	significance
Week	($x10^8$)			($x10^8$)			($x10^8$)		
0	na	na	na	na	na	na	na	na	na
1	0.00	0.00	ns	0.00	0.00	ns	0.01	0.00	ns
2	0.03	0.00	ns	0.15	0.05	ns	0.45	0.07	5%
3	0.33	0.04	1%	0.36	0.03	ns	1.18	0.16	ns
4	0.01	0.00	ns	0.01	0.00	ns	0.00	0.00	ns
6	0.00	0.00	ns	0.01	0.00	ns	0.01	0.00	ns
10	0.01	0.00	ns	0.01	0.00	1%	0.01	0.00	ns

na = not applicable as no fungi detected

APPENDIX 4.2
Bacterial and fungal numbers for the first 14 weeks of pretreatment

Bacterial numbers;

		commercial			Garotta		
week		rep 1	rep 2	rep 3	rep 1	rep 2	rep 3
	3	3900000	4900000	4400000	10600000	10200000	8800000
		2500000	5000000	4300000	10800000	11000000	8000000
		4400000	4600000	3600000	10200000	12500000	8400000
	6	1040000	1000000	720000	5700000	6800000	6900000
		970000	880000	840000	6300000	6100000	6100000
		1000000	900000	690000	5700000	6700000	6900000
	9	450000	420000	370000	1560000	2010000	1870000
		420000	350000	400000	1690000	1940000	1700000
		380000	380000	450000	1620000	1760000	1600000
	12	300000	230000	340000	1400000	970000	1000000
		260000	180000	330000	1320000	1060000	1150000
		210000	280000	290000	1360000	1180000	1020000
	14	240000	196000	184000	400000	480000	400000
		200000	240000	156000	420000	450000	420000
		234000	176000	167000	480000	520000	460000
14 cold		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
14 warm		240000	196000	184000	400000	480000	400000
		200000	240000	156000	420000	450000	420000
		234000	176000	167000	480000	520000	460000

Significance within pretreatments (i.e. between replicates) for bacterial numbers

	Commercial			1.0g 'Garotta'		
	Mean	SE	significance	Mean	SE	significance
Week	($x10^8$)			($x10^8$)		
3	0.42	0.03	ns	1.01	0.05	1%
6	0.09	0.00	1%	0.64	0.02	ns
9	0.04	0.00	ns	0.18	0.01	ns
12	0.03	0.00	ns	0.12	0.01	1%
14	0.02	0.00	ns	0.04	0.00	0.10%
14 cold	0.00	0.00	na	0.00	0.00	na
14 warm	0.02	0.00	ns	0.04	0.00	0.10%

Fungal numbers;

week		commercial rep 1	commercial rep 2	commercial rep 3	Garotta rep 1	Garotta rep 2	Garotta rep 3
	3	4400000	3900000	3800000	9400000	9000000	8500000
		3600000	4200000	3300000	8200000	9900000	8100000
		4000000	4300000	3700000	9000000	9400000	8200000
	6	880000	760000	560000	5800000	5800000	4700000
		1000000	840000	800000	4800000	6500000	4500000
		920000	880000	750000	5300000	7000000	5400000
	9	580000	560000	560000	1640000	2500000	1670000
		600000	520000	590000	1740000	2080000	1800000
		570000	510000	550000	1560000	2240000	1750000
	12	220000	210000	240000	1400000	1400000	1200000
		250000	230000	290000	1260000	1100000	1020000
		240000	280000	190000	1160000	1150000	1080000
	14	240000	248000	200000	620000	700000	620000
		280000	264000	224000	590000	690000	660000
		240000	260000	200000	650000	680000	700000
14 cold		0	0	0	0	0	0
		0	0	0	0	0	0
		0	0	0	0	0	0
14 warm		240000	248000	200000	620000	700000	620000
		280000	264000	224000	590000	690000	660000
		240000	260000	200000	650000	680000	700000

Significance within pretreatments (i.e. between replicates) for fungal numbers

Week	Commercial Mean ($x10^8$)	Commercial SE	Commercial significance	1.0g 'Garotta' Mean ($x10^8$)	1.0g 'Garotta' SE	1.0g 'Garotta' significance
3	0.39	0.01	ns	0.89	0.02	ns
6	0.08	0.00	ns	0.55	0.03	5%
9	0.06	0.00	ns	0.19	0.01	1%
12	0.02	0.00	ns	0.12	0.00	ns
14	0.02	0.00	5.0%	0.07	0.00	ns
14 cold	0.00	0.00	na	0.00	0.00	na
14 warm	0.02	0.00	5.0%	0.07	0.00	ns

Fungal numbers during the warm period of pretreatment

commercial pretreatment;

time	rep 1a	rep 1b	rep 2a	rep 2b	rep 3a	rep 3b
0h	236000	108000	160000	216000	83000	118000
	180000	124000	100000	216000	85000	120000
	198000	90000	134000	200000	94000	116000
48h	404000	592000	720000	664000	734000	440000
	556000	440000	624000	520000	636000	416000
	640000	568000	536000	608000	560000	440000
96h	4000000	1800000	1200000	700000	1600000	900000
	2800000	2000000	1600000	700000	2200000	1100000
	1100000	1800000	1000000	300000	1400000	800000
144h	3000000	5000000	6000000	4000000	13000000	7000000
	4000000	5000000	5000000	8000000	20000000	8000000
	3000000	4000000	5000000	9000000	11000000	7000000
192h	8000000	6000000	2000000	7000000	4000000	3000000
	17000000	3000000	2000000	5000000	2000000	3000000
	12000000	4000000	3000000	4000000	8000000	8000000
240h	8000000	7000000	4000000	10000000	3000000	11000000
	2000000	6000000	4000000	11000000	6000000	5000000
	5000000	7000000	2000000	7000000	5000000	6000000
288h	7100000	9000000	7600000	9600000	9800000	5100000
	11500000	8500000	7900000	8200000	6900000	4800000
	6400000	13100000	10800000	8000000	5700000	9200000
14 day	5600000	7200000	6400000	14000000	12800000	12400000
	4800000	5800000	5400000	16000000	6800000	9600000
	2700000	6600000	5200000	8800000	9600000	5600000
21d	2000000	2000000	2800000	2800000	4400000	4000000
	2000000	1600000	2000000	3200000	4000000	3600000
	1200000	2000000	4000000	3200000	4000000	4400000
28d	1200000	1200000	6000000	6800000	5200000	2600000
	700000	1100000	5200000	2800000	4200000	8800000
	800000	1200000	4800000	3600000	2600000	3200000
35d	3500000	1300000	3400000	3900000	1800000	2300000
	2300000	1800000	4000000	3000000	2600000	2500000
	2000000	1500000	3600000	6000000	1700000	2700000
42d	1100000	500000	1500000	2500000	1200000	1000000
	100000	400000	1500000	1200000	1000000	600000
	400000	300000	1200000	2600000	1000000	1000000

56d	300000	3000000	2800000	2000000	500000	200000
	3000000	2000000	1800000	2400000	100000	100000
	3000000	1000000	2600000	800000 n		200000
70d	n	n	2000000	1200000 n	n	
	n	n	2000000	1200000	1000000 n	
	n	n	1200000	1500000 n	n	

Garotta pretreatment;

time	rep 1a	rep 1b	rep 2a	rep 2b	rep 3a	rep 3b
0h	54000	112000	153000	198000	192000	160000
	83000	138000	185000	202000	204000	180000
	62000	136000	91000	222000	246000	166000
48h	760000	976000	520000	700000	680000	824000
	650000	968000	420000	550000	360000	984000
	632000	1092000	440000	680000	492000	1088000
96h	1200000	1000000	4900000	1900000	3000000	3300000
	2400000	3000000	4600000	2000000	1100000	1900000
	2000000	1800000	4400000	1700000	1500000	3300000
144h	4000000	6000000	9000000	6000000	2000000	14000000
	9000000	7000000	9000000	2000000	4000000	15000000
	4000000	6000000	10000000	1000000	2000000	24000000
192h	8000000	12000000	12000000	18000000	10000000	8000000
	8000000	8000000	20000000	20000000	14000000	4000000
	9000000	10000000	8000000	10000000	10000000	12000000
240h	9000000	11000000	10000000	13000000	10000000	8000000
	9000000	8000000	12000000	11000000	14000000	8000000
	10000000	10000000	12000000	9000000	12000000	11000000
288h	14500000	8400000	10800000	11800000		8900000
	14100000	9500000	10800000	25000000		8000000
	12200000	6800000	10500000	19400000		10000000
14 day	12000000	12000000	13600000	8000000	12800000	8800000
	17200000	12800000	9600000	14000000	12000000	10400000
	15200000	10400000	10000000	10000000	10800000	10000000
21d	15200000	14400000	3600000	4800000	11200000	2000000
	10800000	15600000	5200000	4000000	8000000	1600000
	8000000	22400000	2800000	5200000	4800000	3200000
28d	5600000	6800000	2400000	4400000	2400000	2700000
	8400000	6000000	3200000	4000000	2000000	3000000
	4800000	5200000	6000000	3200000	2000000	2600000
35d	3700000	2000000	3800000	2000000	3200000	2800000
	2500000	2300000	2900000	2800000	3000000	2600000
	2000000	2500000	1900000	2500000	2400000	1400000
42d	800000	2100000	1300000	1100000	1200000	1400000
	1400000	1800000	1000000	1700000	1600000	700000
	1600000	1400000	1200000	700000	2200000	1500000

56d	1200000	1100000	500000	1400000	600000	900000
	1300000	1600000	900000	1300000	800000	1600000
	2400000	1100000	900000	800000	900000	1300000
70d	1200000	1500000	1000000	3000000	3200000	3500000
	2000000	1000000	1000000	4000000	4000000	2500000
	3500000	1000000	600000	4000000	2500000	3000000

Significance within pretreatments (i.e. between replicates)

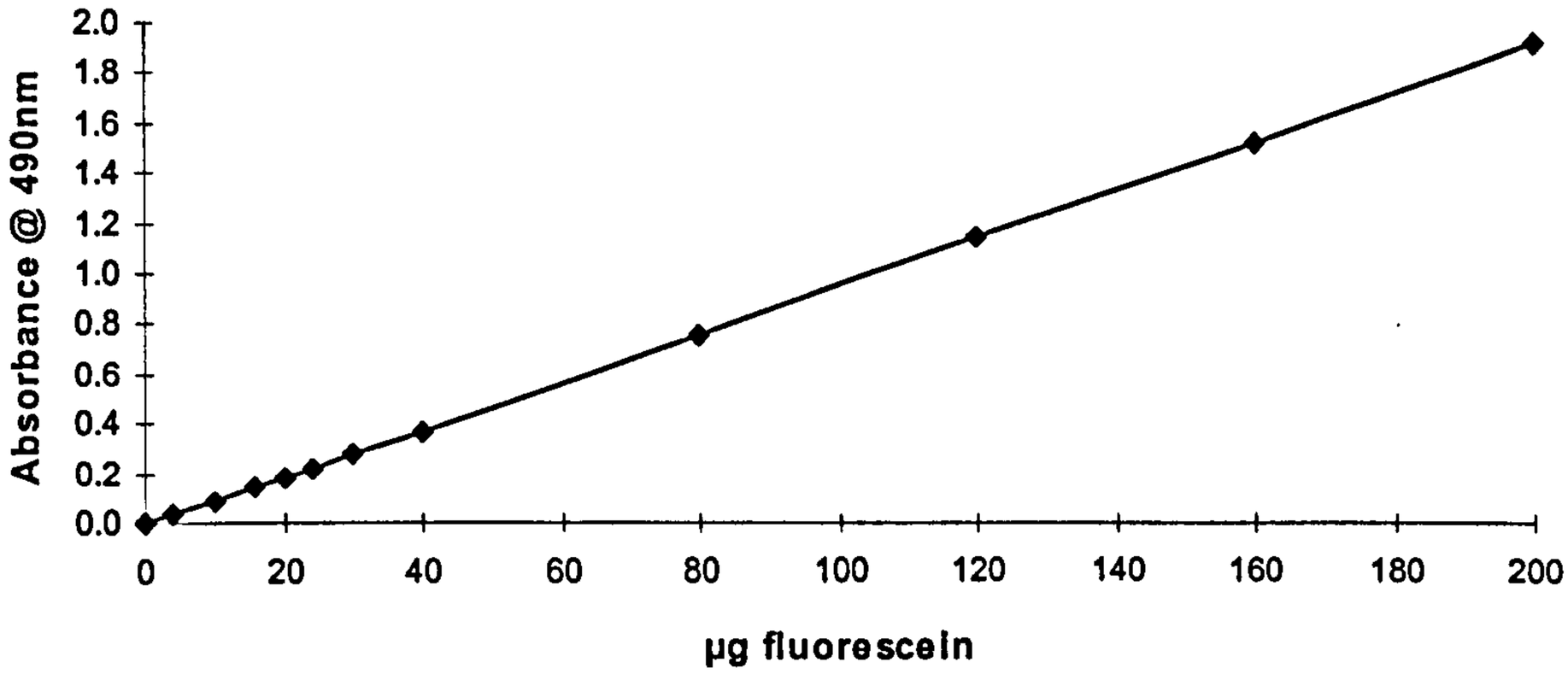
	commercial			Garotta		
time	Mean	SE	significance	Mean	SE	significance
0	138667	20151	0.1%	154667	22439	0.1%
2	558667	18208	ns	712000	15467	0.1%
4	1066667	93425	5.0%	2500000	91583	1.0%
6	6500000	106249	0.1%	7444444	153103	0.1%
8	5611111	107662	5.0%	11166667	142553	ns
10	6055556	87663	ns	10888889	126413	ns
12	5911111	16876	ns	10038889	88247	1.0%
14	6416667	28127	5.0%	11644444	93793	ns
21	3133333	25654	0.1%	7933333	104629	0.1%
28	2700000	105958	5.0%	4150000	237099	1.0%
35	2916667	229352	1.0%	2572222	275458	ns
42	1083333	219393	1.0%	1372222	653314	ns
56	1266667	243899	5.0%	1144444	567332	ns
70	450000	226201	0.1%	2361111	502217	1.0%

APPENDIX 4.3
FDA calibration curve

calibration curve data

ml solution	µg fluorescein	replicate				
		1	2	3	4	average
0	0	0	0	0	0	0.000
0.1	4	*0.031*	*0.031*	0.033	0.031	0.032
0.25	10	0.087	0.087	*0.088*	*0.089*	*0.088*
0.4	16	0.142	0.145	0.142	0.145	0.144
0.5	20	0.186	0.185	0.183	0.17	0.181
0.6	24	0.225	0.222	0.223	0.223	0.223
0.75	30	0.277	0.279	0.277	0.275	0.277
1	40	0.367	0.369	0.372	0.377	0.371
2	80	0.738	0.739	0.747	0.738	0.741
3	120	1.14	1.14	1.122	1.13	1.133
4	160	1.524	1.518	1.521	1.497	1.515
5	200	1.908	1.884	1.908	1.896	1.899

Calibration curve plotted from data above



FDA data for samples

sample	µg fluorescein	replicate				
		1	2	3	4	average
vermiculite	0.00	0.000	0.000	0.000	0.000	0.000
Garotta	1.00	0.008	0.009	0.010	0.008	0.009
dry seed	3.90	0.034	0.028	0.033	0.029	0.031
commercially pretreated seed	0.55	0.004	0.001	0.012	0.004	0.005
Garotta pretreated seed	11.60	0.095	0.115	0.101	0.130	0.110
autoclaved seed	0.13	0.001	0.000	0.000	0.004	0.001
Rhizopus; 2 plugs	8	0.071	0.07	0.074	0.077	0.074
Rhizopus; 4 plugs	40	0.385	0.38	0.38	0.385	0.383

APPENDIX 4.4
FDA assay raw data

1g samples

week	Commercial A	Commercial B	Garotta A	Garotta B
0	0.024	0.032	0.047	0.024
	0.049	0.024	0.039	0.037
	0.036	0.026	0.060	0.022
1	0.216	0.046	0.402	0.247
	0.038	0.080	0.109	0.158
	0.098	0.182	0.539	0.094
2	0.150	0.115	0.412	0.608
	0.122	0.123	0.480	0.407
	0.167	0.143	0.637	0.535
3	0.094	0.121	0.508	0.398
	0.089	0.123	0.458	0.371
	0.114	0.151	0.375	0.333
4	0.165	0.177	0.333	0.496
	0.127	0.132	0.336	0.512
	0.189	0.145	0.321	0.602
5	0.182	0.156	0.441	0.840
	0.137	0.196	0.431	0.700
	0.137	0.179	0.357	0.790
6	0.212	0.223	0.579	0.962
	0.236	0.241	0.603	0.946
	0.135	0.212	0.632	0.918
7	0.199	0.209	0.828	1.052
	0.242	0.333	0.652	1.070
	0.170	0.366	0.671	1.048
8	0.176	0.241	0.820	1.038
	0.232	0.267	0.734	0.931
	0.169	0.353	0.566	0.929
9	0.282	0.292	0.676	0.900
	0.243	0.237	0.914	1.122
	0.292	0.267	0.930	0.915
10	0.242	0.324	0.776	1.010
	0.198	0.378	0.785	1.258
	0.363	0.390	0.679	1.140
11	0.444	0.393	0.724	0.896
	0.529	0.361	0.607	1.153
	0.415	0.406	0.869	1.074
12	0.682	0.337	0.636	0.917
	0.701	0.250	0.682	0.985
	0.316	0.336	0.512	0.797

13	0.407	0.361	0.609	0.704
	0.790	0.348	0.675	0.866
	0.655	0.358	0.726	0.800
14	0.897	0.309	0.780	1.049
	0.823	0.400	0.920	1.003
	0.727	0.413	0.843	0.970
15	0.623	0.362	0.668	0.895
	0.754	0.255	0.723	0.973
	0.703	0.381	0.712	0.928
16	0.588	0.190	0.757	0.933
	0.402	0.273	0.505	0.853
	0.433	0.278	0.566	0.908
18	0.378	0.415	0.894	1.132
	0.495	0.209	0.966	0.822
	0.571	0.202	0.866	1.132
20	0.513	0.374	0.962	0.974
	0.488	0.209	0.797	1.202
	0.560	0.226	0.883	1.234
22	0.273	0.160	0.500	1.004
	0.318	0.241	0.578	0.812
	0.508	0.156	0.470	1.140
24	0.642	0.220	0.658	0.965
	0.622	0.353	0.633	0.930
	0.413	0.219	0.740	0.707

Significance within pretreatments (i.e. between replicates)(data has been converted to µg fluorescein)

	commercial			1.0g Garotta		
Week	Mean	SE	signific	Mean	SE	signific
0	3.35	0.4	ns	4.02	0.6	ns
1	11.59	3.1	ns	27.19	7.7	ns
2	14.39	0.9	ns	54.05	4.2	ns
3	12.15	1.0	ns	42.88	2.8	ns
4	16.41	1.1	ns	45.64	5.1	1%
5	17.32	1.1	ns	62.47	8.9	1%
6	22.10	1.7	ns	81.45	8.0	0.1%
7	26.66	3.4	ns	93.40	8.4	1%
8	25.24	2.9	ns	88.08	7.2	5%
9	28.31	1.0	ns	95.79	6.1	ns
10	33.26	3.4	ns	99.14	9.9	5%
11	44.73	2.5	ns	93.44	8.8	5%
12	46.02	8.6	ns	79.50	7.7	5%
13	51.24	8.1	ns	76.88	3.9	ns
14	62.65	10.8	1%	97.68	4.4	5%
15	54.03	8.9	1%	85.99	5.6	1%
16	37.98	6.1	5.0%	79.38	7.8	5%
18	39.85	6.4	ns	102.02	5.8	ns
20	41.60	6.5	5%	106.23	7.5	ns
22	29.07	5.6	ns	79.06	12.0	5%
24	43.34	8.0	5%	81.32	6.1	ns

cotton squares

week	Commercial A	Commercial B	Garotta A	Garotta B
6	0.019	0.091	0.468	0.513
	0.013	0.024	0.371	0.423
	0.008	0.066	0.279	0.489
7	0.026	0.036	0.517	0.552
	0.033	0.062	0.652	0.688
	0.033	0.054	0.485	0.783
8	0.046	0.057	0.792	0.575
	0.023	0.098	0.479	0.446
	0.042	0.041	0.636	0.543
9	0.045	0.026	0.578	0.370
	0.046	0.041	0.819	0.564
	0.020	0.035	0.772	0.398
10	0.144	0.041	0.543	0.676
	0.137	0.102	0.880	0.694
	0.150	0.053	0.704	0.700
11	0.162	0.058	0.345	0.433
	0.117	0.094	0.720	0.580
	0.168	0.043	0.739	0.351
12	0.135	0.038	0.678	0.428
	0.200	0.050	0.543	0.513
	0.148	0.043	0.504	0.426
13	0.038	0.022	0.039	0.082

	0.034	0.026	0.049	0.067
	0.041	0.028	0.045	0.042
14	0.025	0.025	0.043	0.054
	0.027	0.018	0.061	0.045
	0.018	0.015	0.045	0.052
15	0.006	0.005	0.051	0.044
	0.005	0.006	0.024	0.047
	0.006	0.013	0.038	0.037
16	0.031	0.011	0.037	0.049
	0.004	0.021	0.029	0.037
	0.010	0.039	0.051	0.054
18	0.015	0.030	0.041	0.038
	0.012	0.015	0.037	0.037
	0.014	0.009	0.034	0.036
20	0.031	0.021	0.048	0.045
	0.030	0.021	0.045	0.048
	0.030	0.018	0.038	0.044
22	0.029	0.018	0.039	0.031
	0.030	0.027	0.031	0.033
	0.020	0.012	0.041	0.051
24	0.022	0.027	0.037	0.033
	0.025	0.027	0.030	0.034
	0.026	0.024	0.045	0.047

Significance within pretreatments (data has been converted to µg fluorescein)

	commercial			1.0g Garotta		
Week	Mean	SE	signific	Mean	SE	signific
6	3.88	1.4	ns	44.64	3.7	ns
7	4.28	0.6	ns	65.96	4.9	ns
8	5.39	1.1	ns	60.92	5.4	ns
9	3.74	0.5	ns	61.45	8.0	5%
10	11.00	2.1	5%	73.67	4.6	ns
11	11.27	2.2	5%	55.61	7.6	ns
12	10.78	2.9	1%	54.27	4.0	ns
13	3.32	0.3	5%	5.69	0.7	ns
14	2.25	0.2	ns	5.27	0.3	ns
15	0.72	0.1	ns	4.22	0.4	ns
16	2.04	0.6	ns	4.51	0.4	ns
18	1.67	0.3	ns	3.91	0.1	ns
20	2.65	0.2	0.1%	4.70	0.2	ns
22	2.39	0.3	ns	3.97	0.3	ns
24	2.65	0.1	ns	4.01	0.2	ns

APPENDIX 4.5
Cotton strip data

Cotton strip data for the 13 week experiment comparing the commercial, 1.0g Garotta, vermiculite only and vermiculite + Garotta pretreatments

	vermiculite			vermiculite + Garotta			commercial			1.0g Garotta		
	1	2	3	1	2	3	1	2	3	1	2	3
1	450	455	535	175	245	260	495	550	580	330	480	475
	555	420	505	245	255	200	520	500	540	375	440	475
	455	545	515	275	200	270	495	550	350	415	435	460
	495	545	475	295	275	270	540	530	515	330	410	510
	495	500	475	255	250	220	500	525	530	395	420	380
Mean	490	493	501	249	245	244	510	531	503	369	437	460
2	545	490	530	255	200	210	500	530	390	275	345	270
	510	545	530	220	180	235	460	530	465	290	310	185
	500	520	520	220	170	215	530	545	535	265	250	205
	485	470	530	230	245	240	465	555	485	260	315	225
	520	540	520	205	240	220	480	555	550	200	160	255
Mean	512	513	526	226	207	224	487	543	485	258	276	228
3	560	475	500	380	260	440	515	550	535	330	280	370
	545	515	545	325	170	465	515	370	570	280	345	405
	565	525	515	365	280	400	515	455	535	370	305	380
	530	505	540	375	235	350	530	495	540	300	280	315
	495	505	540	225	225	310	525	520	510	310	295	245
Mean	539	505	528	334	234	393	520	478	538	318	301	343
4	545	525	565	350	295	225	380	440	530	280	355	395
	570	530	565	200	220	250	445	540	505	415	335	445
	515	555	530	285	195	280	495	500	375	280	310	365
	550	550	570	365	275	335	555	540	505	410	375	350
	555	510	560	310	205	290	490	390	395	225	350	370
Mean	547	534	558	302	238	276	473	482	462	322	345	385
5	440	525	520	320	305	420	350	550	450	375	355	410
	515	550	570	315	265	405	380	510	530	350	390	465
	560	485	535	280	285	340	395	465	465	300	440	345
	480	565	560	290	325	360	510	485	480	405	435	360
	515	550	500	345	320	325	505	465	575	335	355	375
Mean	502	535	537	310	300	370	428	495	500	353	395	391
6	515	515	505	300	310	385	365	365	420	425	365	385
	500	565	530	320	215	345	500	520	575	335	385	380
	540	555	525	220	355	320	435	500	350	390	395	375
	520	545	525	320	315	340	440	495	470	380	325	325
	515	570	555	305	235	355	535	455	500	385	410	305
Mean	518	550	528	293	286	349	455	467	463	383	376	354

7	420	510	455	400	325	415	435	480	510	275	350	325
	485	540	540	370	370	435	435	355	350	390	275	390
	460	505	560	310	335	475	475	475	455	365	420	370
	515	535	520	330	350	380	465	335	425	370	390	375
	545	560	515	380	300	390	505	440	400	365	285	375
Mean	485	530	518	358	336	419	463	417	428	353	344	367
8	465	540	525	310	330	380	505	535	510	330	365	340
	490	525	520	305	310	390	365	485	465	415	310	340
	550	465	520	400	265	305	500	525	430	305	345	385
	515	500	490	345	385	315	475	505	325	440	375	260
	510	505	530	310	235	415	465	305	385	350	370	320
Mean	506	507	517	334	305	361	462	471	423	368	353	329
9	575	525	540	220	365	325	500	465	495	370	285	355
	545	555	545	315	380	265	500	465	525	310	290	325
	535	545	460	315	245	415	320	470	545	345	320	375
	545	560	555	340	280	365	500	460	495	360	350	365
	555	445	515	245	360	365	495	365	490	345	350	310
Mean	551	526	523	287	326	347	463	445	510	346	319	346
10	545	535	550	345	220	310	500	515	475	390	335	425
	520	525	550	350	235	315	420	425	470	410	375	370
	515	490	560	385	305	295	530	385	475	400	320	350
	480	525	565	160	335	355	520	425	415	390	400	295
	565	510	555	250	310	235	515	510	530	425	370	355
Mean	525	517	556	298	281	302	497	452	473	403	360	359
12	525	520	560	340	160	360	535	475	430	385	350	405
	490	540	520	345	285	320	370	485	530	335	320	390
	550	515	550	350	330	350	540	315	355	315	415	425
	505	525	525	310	310	340	500	285	305	270	320	385
	555	535	545	300	315	410	515	465	480	340	280	395
Mean	525	527	540	329	280	356	492	405	420	329	337	400
13	515	465	560	520	540	565	525	535	535	500	520	520
	500	505	530	565	550	535	500	525	515	525	520	480
	510	500	540	525	475	540	535	530	465	525	515	505
	535	550	495	530	560	550	495	520	535	495	420	490
	550	555	570	525	550	495	525	535	515	495	535	475
Mean	522	515	539	533	535	537	516	529	513	508	502	494

Levels of significance within pretreatments (i.e. between replicates) for the data on previous page.

Week	vermiculite (- seed) Mean	SE	signif	commercial (+ seed) Mean	SE	signif	1.0g Garotta (- seed) Mean	SE	signif	1.0g Garotta (+ seed) Mean	SE	signif
1	495	10.32	ns	515	13.27	ns	246	8.61	ns	422	13.89	5%
2	517	5.75	ns	505	12.13	ns	219	6.10	ns	254	13.28	ns
3	524	6.59	ns	512	12.14	ns	320	22.30	5%	321	11.62	ns
4	546	5.11	ns	472	16.27	ns	272	14.18	ns	351	15.02	ns
5	525	9.54	ns	474	16.05	ns	327	11.18	5%	380	11.49	ns
6	532	5.64	5%	462	17.09	ns	309	12.91	ns	371	8.75	ns
7	511	10.48	ns	436	14.14	ns	371	12.44	5%	355	11.54	ns
8	510	6.38	ns	452	18.99	ns	333	13.32	ns	350	11.56	ns
9	533	9.27	ns	473	14.99	ns	320	14.90	ns	337	7.38	ns
11	533	6.83	5%	474	12.51	ns	294	15.86	ns	374	9.76	ns
12	531	5.07	ns	439	23.08	ns	322	13.88	ns	355	12.57	ns
13	525	7.63	ns	519	5.07	ns	535	6.47	ns	501	7.39	ns

data for the comparison of the 1.0g Garotta, 0.5g Garotta and commercial pretreatments

	1.0g Garotta			0.5g Garotta			commercial		
week	1	2	3	1	2	3	1	2	3
1	525	595	595	570	475	590	560	575	575
	560	560	555	495	530	580	580	575	600
	545 md		560	510	570	535	525	565	580
	530	525	575	535	570	520	595	580	565
	435	575	560	515	575	575	555	580	565
mean	519	564	569	525	544	560	563	575	577
2	360	460	345	460	455	515	420	590	575
	460	430	465	510	500	475	440	550	590
	455	515	435	405	465	495	545	450	525
	420	400	425	500	440	460	455	565	545
	440	375	430	525	335	505	535	535	560
mean	427	436	420	480	439	490	479	538	559
3	350	385	435	435	*455*	*465*	*555*	*415*	*500*
	250	265	400	435	360	380	490	495	545
	245	240	235	315	385	460	460	395	555
	245	415	350	410	425	405	565	390	490
	235	270	315	430	475	435	560	535	540
mean	265	315	347	405	420	429	526	446	526
4	380	225	420	410	335	425	545	350	465
	425	340	340	465	350	360	515	430	540
	375	405	350	415	320	260	475	510	415
	385	365	385	245	350	280	545	545	550
	225	295	295	425	330	320	585	550	515
mean	358	326	358	392	337	329	533	477	497
5	270	360	365	365	315	440	540	370	460
	250	365	270	440	390	400	460	445	420
	325	375	430	370	345	415	525	505	510
	360	305	370	305	300	425	510	505	355
	385	325	380	390	410	395	530	485	390
mean	318	346	363	374	352	415	513	462	427
6	295	420	455	410	380	475	470	470	415
	420	360	305	425	465	460	375	465	440
	320	330	420	475	385	450	435	440	450
	330	440	475	380	340	375	435	435	315
	425	440	445	435	405	465	450	460	425
mean	358	398	420	425	395	445	433	454	409
7	350	535	405	495	410	510	470	505	545
	440	515	470	500	475	445	520	370	520
	435	350	360	475	405	460	490	520	540
	430	380	445	495	445	500	535	490	545
	390	395	465	495	470	415	530	480	485
mean	409	435	429	492	441	466	509	473	527

8	440	425	455	315	485	520	555	500	510
	495	365	395	485	545	490	450	540	555
	505	445	470	480	460	520	485	510	600
	485	495	325	490	535	515	525	530	545
	500	495	495	455	485	465	540	575	420
mean	485	445	428	445	502	502	511	531	526
9	400	420	350	420	500	500	495	430	505
	435	485	330	485	420	415	500	500	550
	440	480	360	350	470	475	465	485	555
	445	340	310	420	415	480	525	510	495
	295	365	470	425	285	420	515	550	445
mean	403	418	364	420	418	458	500	495	510
11	430	445	425	395	440	405	570	480	500
	425	415	470	345	470	555	440	500	355
	260	440	435	485	515	205	490	465	455
	440	225	395	540	370	480	510	405	530
	415	465	485	485	475	485	495	440	565
mean	394	398	442	450	454	426	501	458	481
12	360	480	405	420	360	430	575	550	480
	450	475	330	435	445	485	565	380	530
	445	365	350	500	410	530	515	525	420
	470	435	420	430	525	485	520	520	405
	365	485	300	540	510	515	525	515	495
mean	418	448	361	465	450	489	540	498	466

levels of significance within pretreatments (i.e. between replicates) for the data above

	commercial			1.0g 'Garotta'			0.5g 'Garotta'		
Week	Mean	SE	signif	Mean	SE	signif	Mean	SE	signif
1	572	4.57	ns	551	9.96	ns	543	9.11	ns
2	525	14.50	ns	428	11.36	ns	470	12.75	ns
3	499	15.57	0.05	309	18.95	ns	418	11.21	ns
4	502	16.45	ns	347	16.20	ns	353	16.73	ns
5	467	15.47	ns	342	12.98	ns	380	11.95	ns
6	432	10.43	ns	392	15.82	ns	422	11.12	ns
7	503	11.44	ns	424	14.57	ns	466	9.08	ns
8	523	11.96	ns	453	14.11	ns	483	13.85	ns
9	502	9.36	ns	395	16.38	ns	432	14.99	ns
11	480	14.66	ns	411	18.74	ns	443	22.98	ns
12	501	14.80	ns	409	15.52	ns	468	13.75	ns

data for the comparison between the commercial and Garotta pretreatments over the entire pretreatment period

		Commercial		Garotta	
		1	2	1	2
1		515	385	500	485
		550	510	455	435
		535	550	420	290
		530	535	435	485
		570	530	485	465
mean		540	502	459	432
2		510	410	270	320
		485	560	280	265
		510	420	245	275
		545	475	305	285
		475	525	220	260
mean		505	478	264	281
3		420	495	300	330
		425	465	290	245
		465	455	285	310
		495	485	280	300
		505	540	205	270
mean		462	488	272	291
4		450	495	245	305
		465	495	260	250
		535	540	335	285
		515	310	270	295
		405	365	295	295
mean		474	441	281	286
	5	530	515	350	265
		520	460	300	285
		530	545	245	325
		470	415	235	330
		450	495	325	345
	mean	500	486	291	310
	6	520	545	350	420
		495	530	275	400
		480	525	285	315
		550	375	300	310
		535	510	300	275
mean		516	497	302	344
		485	530	345	350
8		515	550	260	315
		545	535	310	325
		475	395	200	340
		510	570	360	375
		506	516	295	341

mean				
10	515	555	330	405
	530	440	395	455
	485	515	375	400
	525	565	425	360
	535	515	275	395
mean	518	518	360	403
12	460	560	255	360
	470	450	345	365
	455	510	300	345
	445	410	255	295
	435	465	295	370
mean	453	479	290	347
13	510	480	535	520
	530	535	535	510
	540	520	520	495
	520	520	490	525
	510	510	550	540
mean	522	513	526	518
14	500	585	525	500
	555	545	540	580
	545	515	470	555
	565	540	560	545
	565	515	500	495
mean	546	540	519	535
15	545	520	490	555
	570	565	560	510
	525	545	495	545
	535	580	505	475
	575	525	525	525
mean	550	547	515	522
16	515	535	545	535
	565	485	550	520
	530	560	565	540
	560	575	530	530
	555	550	530	535
mean	545	541	544	532
18	510	525	370	520
	550	535	535	540
	510	525	450	520
	565	550	525	530
	540	555	480	475
mean	535	538	472	517

	20	570	495	520	525
		535	565	520	545
		585	540	505	530
		485	550	550	570
		515	495	555	550
mean		538	529	530	544
	22	555	500	535	500
		535	455	525	500
		585	475	525	535
		565	525	565	515
		565	560	560	505
mean		561	503	542	511
	24	555	535	485	525
		495	490	480	445
		495	475	465	495
		535	495	535	495
		440	540	495	515
mean		504	507	492	495

levels of significance within pretreatments (i.e. between replicates) for the data above

	commercial			1.0g 'Garotta'		
Week	Mean	SE	significance	Mean	SE	significance
1	521	16.09	ns	446	19.20	ns
2	492	15.53	ns	273	8.98	ns
3	475	11.62	ns	282	11.18	ns
4	458	24.06	ns	284	8.73	ns
5	493	13.42	ns	301	13.07	ns
6	507	16.13	ns	323	16.13	ns
8	511	15.83	ns	318	16.55	ns
10	466	13.22	ns	319	13.93	5%
12	474	14.08	0.1%	341	15.66	ns
13	518	10.21	ns	522	9.84	ns
14	543	8.37	ns	527	11.01	ns
15	549	7.11	ns	519	9.04	ns
16	543	8.60	ns	538	4.03	ns
18	537	5.97	ns	495	16.76	ns
20	534	11.03	ns	537	6.33	ns
22	532	13.56	5%	527	7.27	5%
24	506	11.14	ns	494	8.57	ns

APPENDIX 5.1
Germination data - *Rosa corymbifera* 'Laxa' laboratory

germination data for the laboratory tests on *Rosa corymbifera* 'Laxa'

	number germinated replicate			
	1	2	3	4
commercial	13	4	12	12
0.5g Garotta	86	78	78	82
1.0g Garotta	84	98	99	90

germination data for the pretreatments with nitram and gem superphosphate

	number germinated (number viable) replicate					
	1	2	3	4	5	6
commercial	1 (100)	2 (100)	4 (100)	0 (100)	2 (100)	3 (100)
1.0g Garotta	96 (100)	96 (100)	95 (100)	94 (100)	96 (100)	94 (100)
nitram	8 (100)	6 (100)	4 (100)	3 (100)	1 (100)	2 (100)
Gem	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
nitram + gem	22 (100)	26 (100)	24 (100)	34 (100)	38 (100)	32 (100)

germination data for the pretreatments with nitram and gem superphosphate using surface sterilised seed

	number germinated (number viable) replicate					
	1	2	3	4	5	6
commercial	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
1.0g Garotta	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
nitram	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
Gem	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
nitram + gem	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)

germination data for the 12 week warm and 6 week warm periods of incubation (also the addtion of Garotta weekly)

	number germinated replicate							
	1	2	3	4	5	6	7	8
Commercial	3	4	2	5	6	1	3	4
1.0g 'Garotta'	68	73	86	74	89	88	77	79
1.0g 'Garotta' (6 week warm)	78	79	84	82	79	73	82	81
Weekly 'Garotta' (6 week warm)	86	86	82	81	80	85	79	80

APPENDIX 5.2
Laboratory germination data - other species

germination data for the laboratory trials on the other native tree species

		number germinated															
		replicate															
		1a	1b	1c	1d	2a	2b	2c	2d	3a	3b	3c	3d	4a	4b	4c	4d
Acer	commercial	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
campestre	0.5g Garotta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1.0g Garotta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Crataegus	commercial	6	4	5	4	4	5	5	5	5	7	4	5	4	0	0	0
monogyna	0.5g Garotta	7	7	4	6	6	10	3	6	5	8	7	5	4	4	7	7
	1.0g Garotta	10	13	13	9	12	11	10	14	13	9	9	10	11	9	14	11
Prunus	commercial	6	4	2	2	5	5	7	5	4	5	3	5	6	6	6	5
spinosa	0.5g Garotta	7	8	8	11	10	9	8	9	10	9	7	9	8	11	6	7
	1.0g Garotta	11	13	13	11	9	8	9	11	8	10	8	9	9	12	9	11
Rosa	commercial	8	11	5	9	12	7	6	13	12	9	5	8	9	10	9	11
canina	0.5g Garotta	48	51	41	39	39	48	40	43	38	37	36	44	41	47	49	47
	1.0g Garotta	28	34	32	34	30	31	31	36	30	35	33	35	36	33	31	36
Tilia	commercial	1	0	1	0	2	1	0	1	1	0	1	0	0	1	1	0
platyphyllos	0.5g Garotta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1.0g Garotta	1	3	0	0	1	1	0	1	0	0	2	2	0	0	2	0

APPENDIX 5.3
Field trial layout

Layout of the field trial at Oakover Nurseries.

	seed bed 1	seed bed 2
block 1	3.3.1	1.3.1
	3.2.1	1.4.1
	2.4.1	2.3.1
	3.1.1	2.1.1
	1.1.1	3.4.1
	2.2.1	1.2.1
block 2	2.2.2	2.3.2
	2.1.2	3.2.2
	2.4.2	3.1.2
	3.3.2	1.2.2
	3.4.2	1.4.2
	1.3.2	1.1.2
block 3	2.3.3	1.1.3
	2.1.3	3.3.3
	3.2.3	3.4.3
	1.3.3	1.2.3
	2.4.3	3.1.3
	1.4.3	2.2.3
block 4	3.3.4	3.1.4
	2.1.4	1.1.4
	1.2.4	3.2.4
	2.3.4	2.2.4
	1.3.4	1.4.4
	3.4.4	2.4.4

	seed bed 1	seed bed 2
block 5	3.3.5	1.4.5
	3.4.5	2.3.5
	2.4.5	1.1.5
	2.1.5	3.2.5
	1.2.5	1.3.5
	2.2.5	3.1.5
block 6	3.3.6	1.1.6
	1.2.6	2.4.6
	1.3.6	2.2.6
	3.2.6	3.4.6
	2.3.6	3.1.6
	1.4.6	2.1.6
block 7	2.1.7	1.4.7
	2.3.7	1.2.7
	3.2.7	2.2.7
	1.1.7	3.4.7
	3.1.7	2.4.7
	1.3.7	3.3.7
block 8	2.1.8	2.3.8
	1.4.8	3.3.8
	3.4.8	3.1.8
	1.3.8	3.2.8
	2.2.8	1.2.8
	2.4.8	1.1.8

Each block consisted of the two seed beds and six consecutitive rows. The replicates of each pretreatment were randomly placed across the whole of the two beds. The first digit of each position (**X**.x.x) represents the pretreatment, i.e. 1 = commercial pretreatment, 2 = 0.5g 'Garotta' pretreatment and 3 = 1.0g 'Garotta' pretreatment.

The second digit (x.**X**.x) represents the replicate number from each pretreatment. Four replicates were sampled so this is either 1, 2, 3 or 4. Finally the third digit (x.x.**X**) represents the subsample from each replicate. Eight lots of 50 seeds were sampled (total of 400) from each replicate. These subsamples were placed in the same block each time, thus block 1 contained all the x.x.1 subsamples, block 2 contained all the x.x.2 subsamples etc. Initially six replicates for each pretreatment for each species were setup. This was to allow for 4 replicates to be randomly selected at the end of the pretreatment incubation for germination trials. The 2 extra replicates were as precautions in case of damage to any of the replicates.

APPENDIX 5.4
Rosa corymbifera 'Laxa' field trial data

germination data for the field trial on *Rosa corymbifera* 'Laxa'

	number germinated (out of 50) replicate							
	1	2	3	4	5	6	7	8
1.1.x	7	12	11	9	9	8	15	10
1.2.x	5	10	7	5	11	8	10	12
1.3.x	8	9	12	10	11	14	10	12
1.4.x	12	8	19	11	11	15	12	10
2.1.x	28	33	35	42	45	48	36	32
2.2.x	34	44	36	32	45	41	29	38
2.3.x	31	50	36	37	30	36	30	39
2.4.x	38	42	34	40	38	35	36	35
3.1.x	42	34	37	40	35	36	36	36
3.2.x	48	38	42	45	41	39	49	44
3.3.x	38	46	47	38	50	36	36	29
3.4.x	47	36	44	42	46	41	37	34

APPENDIX 5.5
Field trial data - other species

Crataegus monogyna

	number germinated (out of 50) replicate							
	1	2	3	4	5	6	7	8
1.1.x	29	24	28	21	22	29	16	26
1.2.x	25	25	26	30	19	21	21	32
1.3.x	26	26	20	24	19	25	24	27
1.4.x	22	18	26	25	21	21	27	22
2.1.x	28	31	25	35	19	23	30	21
2.2.x	33	27	21	26	29	29	35	27
2.3.x	26	24	25	29	22	23	28	30
2.4.x	26	23	28	30	29	25	24	26
3.1.x	31	26	28	28	23	23	30	27
3.2.x	37	26	30	33	29	31	24	28
3.3.x	32	28	27	20	28	27	29	27
3.4.x	26	25	22	29	23	28	25	30

Rosa canina

	number germinated (out of 50) replicate							
	1	2	3	4	5	6	7	8
1.1.x	4	6	13	9	9	11	14	11
1.2.x	3	8	5	7	4	4	8	8
1.3.x	6	4	7	5	4	3	7	17
1.4.x	10	18	15	16	11	10	17	12
2.1.x	34	31	41	31	21	34	38	37
2.2.x	31	31	29	34	21	31	39	28
2.3.x	33	37	35	30	29	35	23	37
2.4.x	30	25	38	32	18	33	30	29
3.1.x	24	35	35	32	31	35	37	34
3.2.x	32	31	35	35	31	26	27	32
3.3.x	38	38	36	32	26	29	35	29
3.4.x	37	39	41	27	31	38	40	4

Prunus spinosa

	number germinated (out of 50) replicate							
	1	2	3	4	5	6	7	8
1.1.x	16	19	20	20	21	23	24	23
1.2.x	18	17	16	16	16	14	15	16
1.3.x	22	20	22	18	16	13	22	22
1.4.x	16	18	19	20	17	20	18	17
2.1.x	32	23	27	26	25	24	21	26
2.2.x	22	27	23	25	26	26	27	21
2.3.x	26	25	18	27	23	20	31	18
2.4.x	23	28	26	25	22	24	19	22
3.1.x	29	25	20	31	28	20	25	25
3.2.x	26	22	18	20	26	22	31	22
3.3.x	28	22	21	17	21	23	20	23
3.4.x	27	20	23	22	21	23	26	20

Tilia platyphyllos

	number germinated (out of 50) replicate							
	1	2	3	4	5	6	7	8
1.1.x	4	3	6	4	8	2	3	3
1.2.x	8	8	3	7	14	2	9	6
1.3.x	1	8	6	6	14	4	6	10
1.4.x	5	7	3	6	4	3	6	3
2.1.x	5	6	3	2	8	7	5	6
2.2.x	5	4	5	9	5	6	6	4
2.3.x	3	4	4	3	7	2	7	6
2.4.x	5	6	5	4	7	8	2	6
3.1.x	9	6	4	6	7	2	7	8
3.2.x	3	3	3	5	6	10	5	3
3.3.x	4	3	5	6	5	7	7	6
3.4.x	3	4	0	6	3	3	5	8

Acer campestre

	number germinated (out of 50) replicate							
	1	2	3	4	5	6	7	8
1.1.x	0	0	0	0	0	0	0	0
1.2.x	0	0	0	0	0	0	0	0
1.3.x	0	0	0	0	0	0	0	0
1.4.x	0	0	0	0	0	0	0	0
2.1.x	0	0	0	0	0	0	0	0
2.2.x	0	0	0	0	0	0	0	0
2.3.x	0	0	0	0	0	0	0	0
2.4.x	0	0	0	0	0	0	0	0
3.1.x	0	0	0	0	0	0	0	0
3.2.x	0	0	0	0	0	0	0	0
3.3.x	0	0	0	0	0	0	0	0
3.4.x	0	0	0	0	0	0	0	0