

MICROPROPAGATION AND MICROGRAFTING OF PISTACHIO
(Pistacia vera L. and Pistacia atlantica Desf.)

by

ABDELHADI ABOUSALIM

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Wye College

University of London

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ABSTRACT

In vitro propagation and micrografting techniques of pistachio were developed on two Pistacia species of direct relevance to Morocco (P. vera and P. atlantica).

Successful in vitro establishment was achieved for seeds of P. vera (98%), P. atlantica (89%) and nodal explants of actively growing shoots of four-year-old P. vera grafted plants (93%). In the case of 30-year-old field-grown P. vera trees, 85% survival of nodal explants was obtained with summer-harvested shoots. However, the subsequent maintenance of cultures was difficult because of a slow decline in growth, increased levels of vitrification and chlorosis. Reduction of apical dominance and stimulation of multiple shoot formation was achieved by culturing P. vera and P. atlantica germinating seeds in the presence of BAP (> 4 and $> 0.5 \text{ mg l}^{-1}$, respectively).

In Stage II cultures, MS medium was superior for shoot growth and multiplication but induced faint chlorosis on leaves and callus development at the bases of shoots. BAP at 4 mg l^{-1} was optimal for multiplication of materials derived from seedlings and four-year-old P. vera. However, vitrification tended to increase with the BAP level used. In the case of explants obtained from P. atlantica seedlings, 1 mg l^{-1} BAP was optimal. Growth and multiplication were clearly improved by incubating cultures of four-year-old P. vera at $25^{\circ}\text{C}/16\text{h}$ and were even better at $29^{\circ}\text{C}/12\text{h}$ as opposed to $19^{\circ}\text{C}/16\text{h}$. Shoot-tip necrosis (STN) persisted in cultures of both species at Stages I, II and III. Evidence was obtained that STN was associated with fast growing and low transpiring shoot-tips. The results obtained showed it was most likely to be a calcium- rather than a boron-related physiological disorder aggravated by low vapour pressure deficit in culture vessels. Supplementation of media with calcium gluconate to semi-solid culture media reduced development of STN but did not prevent it. A practical solution to this problem was developed whereby periodic inversion of tubes containing a liquid medium (supplemented with $15 \text{ mM Ca gluconate}$) prevented development of STN in cultures.

In Stage III, best rooting of P. vera and P. atlantica seedling materials was achieved with explants previously subcultured only once on Stage II and previously induced to root (83.3 and 87.5%, respectively) on 2 and 1 mg l^{-1} IBA. Lower rooting responses were obtained for four-year-old-derived shoots of P. vera (50%) by a 1000 mg l^{-1} IBA dip for 10 s.

At Stage IV, 81.8% in vitro rooted shoots of P. vera seedling materials were successfully weaned in vivo. Direct rooting of 100% P. vera seedling explants was achieved following a 500 mg l^{-1} IBA dip for 5 s.

Pistachio micrografting was investigated in vitro as well as in vivo. In the former case a 'Milcap' system proved crucial for obtaining well developed micrografts in vitro. High levels of graft take were achieved with 10 mm (100%) and 1 - 3 mm (83 - 92%) long scions obtained from Stage II cultures of four-year-old P. vera. Only 33.3% micrografts survived when 0.5-0.7 mm long scions were used and no response was obtained with meristem-tips of <0.3 mm in length. In vivo micrografting of four-year-old P. vera derived shoots onto seven-week-old P. vera seedlings was attempted and 83% take was achieved. Light and fluorescent microscopy investigations showed that cell divisions occurred as early as three days after micrografting in vitro and that vascular continuity was established across grafts by three weeks after micrografting.

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LIST OF ABBREVIATIONS

%	percentage	min	minute
°C	degree celsius (centigrade)	ml	millilitre
2iP	6- ~~X~~ - ~~X~~ -dimethylaminopurine	mM	millimolar
A	Anderson medium	mm	millimetre
AA	ascorbic acid	MS	Murashige and Skoog medium
B	boron	MSF	multiple shoot formation
BAP	6-benzylaminopurine	n/No	number
c.	approximately	nd	not determined
Ca	calcium	NFT	nutrient film technique
CA	citric acid	nm	nanometre
CC	activated charcoal	NT	<u>Nictotiana tabacum</u> L.
DDW	doubled deionised water	P	probability
df	degrees of freedom	p/pp	page/page(s)
e.g.	for example	PVP	polyvinylpyrrolidone
ed(s)	editor(s)	RH	relative humidity
<u>et al</u>	and others	rpm	revolution per minute
F	Fisher test	s	second
FM	fluorescent microscopy	SA	<u>Solanum aviculare</u> Forst
GA ₃	Gibberellic acid	SE	standard error
g l ⁻¹	gramme per litre	SEM	scanning electron microscopy
h	hour	SQR	square root transformation
i.e.	that is	STN	shoot-tip necrosis
IBA	3-indolebutyric acid	TEM	transmission electron microscopy
K	Knop medium	µm	micrometre
KPa	KiloPascal	µM	micromolar
LG	longitudinal section	UV	ultra-violet
LM	light microscopy	V/V	volume per volume
Log _e	logarithm to base e transformation	VPD	vapour pressure deficit
LSD	least significant difference	vs	versus
lx	lux	WPM	woody plant medium
mb	millibar	X ²	chi-square test
mg l ⁻¹	milligramme per litre		

CHAPTER ONE

INTRODUCTION

CHAPTER ONE : INTRODUCTION

Pistachio is an important nut tree of arid and semi-arid regions. In Morocco, P. vera (which is the only species with edible nuts) was introduced in the 1950's to test its adaptation to the local conditions. Commercial orchards were established in the 1970's following the promising results provided by the first trials of acclimatization of this species. P. vera cv. Mateur is at the moment the main cultivar used for commercial purposes and it is usually grafted either onto P. vera or P. atlantica seedling rootstocks. The development of the large arid and semi-arid area existing across the country, and the need for preserving soils against erosion are the main objectives of the pistachio plantation programme in Morocco. Nowadays, the major limitation facing the widespread expansion of pistachio plantations is the shortage of plant material and this has been made more acute by difficulties in propagating this species using the conventional method of grafting. One of the approaches to solving this problem of supply of plant material is to use in vitro techniques from which pistachio could benefit just as other species have already done.

The original objectives of the present study were, therefore, as follows:

- a) to extend the existing micropropagation strategies available for different Pistacia species to seedling materials of P. vera and P. atlantica used as rootstocks in Morocco;
- b) to develop micropropagation methods which could also be used on mature materials of P. vera cv. Mateur in order to produce scions;
- c) to develop a workable micrografting system for pistachio. Such systems would benefit this species as they have already done for other fruit species. The aim was to provide a model system which might be used to rejuvenate and/or invigorate selected mature scions which are notorious for their recalcitrant behaviour in response to conventional propagation procedures;

- d) to test the effects of micrografting mature scions of P. vera onto seedlings or onto rooted micropropagated shoots on the relative levels and rates of rejuvenation and/or invigoration achievable by repeated grafting in vitro and in vivo. However, this original objective and the experimental work which would have been involved proved impossible to carry out due to the extent of various problems associated with the supply as well as the manipulation of mature shoot materials of P. vera cv. Mateur which were realised during the early phases of this study.

The work reported in this thesis was conducted in Morocco and in Britain as a joint research programme between the Department of Horticulture of the 'Institut Agronomique et Vétérinaire Hassan II' in Rabat and the Unit for Advanced Propagation Systems of Wye College (University of London).

CHAPTER TWO

LITERATURE REVIEW



PLATE 1 : Mature pistachio tree (P. vera cv. Mateur) growing in the Meknes area, Morocco.

CHAPTER TWO : LITERATURE REVIEW

2.1 PROPAGATION OF PISTACHIO

2.1.1 Introduction

The pistachio is a tree species within the family Anacardiaceae (Plate 1). It is a dioecious nut tree, and is wind pollinated. Pistacia vera is the only species with edible nuts (Joley, 1979), and is probably native to central Asia (Whitehouse, 1957). In 1985, the world production of pistachios was 127,000t. Asia was the principal production area with 85% of global world production, and the major producers were Iran (55%) and Turkey (20%) (Anon, 1985). The average annual yield per tree, over 12-15 years of production, ranged from 56.5 Kg (fresh weight) for Kerman to 11 Kg for Red Aleppo cvs. (Joley, 1979). Pistachio nutmeats have high oil (48.3 to 58.3%) and protein (19.41 to 28.88%) contents, and are relatively low in sugar (6.1 to 8.4%) depending on cultivars (Bloch and Brekke, 1960).

The pistachio tree has been reported to be very xerophilous and gypso-calciphilous (Woodroof, 1979). Chilling should be sufficient to break bud dormancy (Woodroof, 1979; Crane and Iwakiri, 1981), and long, hot and dry summers are essential for maturation of their nuts (Woodroof, 1979). Pistachio roots are sensitive to excess water in soil (Joley, 1979). Well drained sandy loams with high lime content are therefore preferred, although many kinds of soils have been reported to be convenient for its culture (Joley, 1979; Woodroof, 1979). Once flower buds start to appear fewer vegetative buds and more flower buds are produced (Crane and Iwakiri, 1981). A strong apical dominance has also been reported to characterize developmental patterns of vegetative and floral pistachio organs (Crane and Iwakiri, 1985). Like other fruit trees, pistachio is known to produce little or no crop after a heavy crop year, i.e., alternate bearing (Woodroof, 1979; Crane and Iwakiri, 1981).

2.1.2 Conventional Pistachio Tree Propagating Techniques

Pistachio is the most difficult of the nut trees to propagate (Joley, 1979). This is normally achieved by grafting or budding mature scions onto seedling rootstocks due to difficulties in rooting cuttings.

2.1.2.1 Propagation by cuttings

Juvenile cuttings taken from Pistacia seedlings have been easily rooted (Joley and Opitz, 1971; Pair and Khatamian, 1982). Softwood cuttings of one-year-old P. chinensis seedlings treated with 5,000 mg l⁻¹ IBA resulted in 92% rooting, and no improvement was observed even when higher IBA concentrations (10,000 and 20,000 mg l⁻¹) were used (Pair and Khatamian, 1982). In contrast, propagation of adult material was difficult. Rooting of adult hardwood and softwood cuttings of two P. vera cultivars and of two clonal rootstocks (P. atlantica and P. palestina) treated with up to 10,000 mg l⁻¹ IBA was unsuccessful (Assaf, 1971). A similar result was obtained with hardwood cuttings of P. chinensis using up to 20,000 mg l⁻¹ IBA (Pair and Khatamian, 1982). In 1960, cuttings taken from mature pistachio trees were rooted using a mist system. However, no more than 5% rooting was achieved (Joley, 1960; Joley and Opitz, 1971). Recently, softwood P. vera cuttings were successfully rooted under a mist system after very high auxin concentrations were used : 35,000 mg l⁻¹ IBA was found to be optimal, and 85% rooting was obtained after six weeks from planting (Al Barazi and Schwabe, 1982). This was an unusually high level of auxin since normally only levels as high as 500 to 10,000 mg l⁻¹ are used for rooting cuttings (Hartmann and Kester, 1983).

2.1.2.2 Propagation by budding

Several different Pistacia species are used as rootstocks. Seedlings of P. atlantica and P. terebinthus are widely used for commercial production (Joley and Opitz, 1971; Woodroof, 1979; Hartmann and Kester, 1983). Top worked cultivars on these two rootstocks quickly outgrow and outyield those grafted onto P. vera in spite of the characteristically slow growth in the nursery of the two former rootstocks (Joley, 1979). Unfortunately, these two stocks have been

reported to be highly susceptible to Verticillium (Hartmann and Kester, 1983). P. integerrima is being used in Verticillium-infected areas because of its resistance to this wilt disease (Hartmann and Kester, 1983; Schubert and Martinelli, 1988). Concerning root knot nematode susceptibility, seedling progeny of P. vera x P. atlantica and P. vera x P. integerrima have been found to be highly resistant as compared to P. vera; P. vera x P. terebinthus seedlings proved to be the least resistant (Joley and Whitehouse, 1953). However, P. terebinthus was reported to be tolerant to Phytophthora spp (Pontikis, 1977).

In order to avoid insect infestation during storage and the inhibition of pistachio seeds, the epicarps (or hulls) are removed just after harvest (Joley and Opitz, 1971; Joley, 1979). The main pre-germination treatments recommended to hasten germination and to increase germination rate are as follows :

- a) Acid scarification with concentrated sulphuric acid in the case of seeds with hard and thick shells (Joley, 1979; Shekafandeh and Shaybany, 1986).
- b) Soaking seeds in water and stratification at low temperature such as in the case of P. vera (4°C for two weeks) (Joley and Opiz, 1971; Chahbar and Dekkaki, 1984), P. terebinthus (5-10°C for six weeks) (Hartmann and Kester, 1983) and P. atlantica (4°C for one month) (Taghzouti, 1984).
- c) Seed treatment with gibberellic acid such as in the case of P. vera (N'Ait M'Barek, 1984) and P. terebinthus (Shekafandeh and Shaybany, 1986).

Seeds are best germinated at 21°C or lower. Higher temperatures result in poor or no germination (Joley and Opitz, 1971; Joley, 1979). Pistacia rootstocks are generally grown in containers because of difficulties in handling and transplanting bare-rooted nursery trees (Joley and Opitz, 1971; Joley and Opitz, 1979; Woodroof, 1979; Ibrahim and Nahlawi, 1983).

T-budding (either upright or inverted) is the most common pistachio

p. 2
propagating technique. The rootstock diameter should be large enough to accommodate pistachio buds which are broad and large as compared with most fruit tree buds (Joley, 1979; Woodroof, 1979). Active growing rootstocks which have well-slipping bark give the best results, with an improvement through the summer and fall (Joley and Opitz, 1971; Al Barazi and Schwabe, 1982; Hartmann and Kester, 1983).

2.1.3 In vitro Propagation of Pistachio Trees : Practices and Problems

Micropropagation of several Pistacia species such as P. vera (Barghchi and Alderson, 1983; Barghchi and Martinelli, 1984; Barghchi and Alderson, 1985; Martinelli, 1988), P. atlantica (Martinelli, 1988), P. terebinthus (Pontikis, 1985) and P. integerrima (Martinelli, 1988) has recently been investigated by several research workers using juvenile and/or mature plant materials or seedling and/or commercial cultivars.

2.1.3.1 STAGE I : Culture Establishment (+)

Surface sterilization of seeds and one to two-year-old glasshouse grown plants is reported to be achieved in two steps: first in 70% ethanol for 45s, and then in 20% commercial bleach solution (sodium hypochlorite) for 25 min (Barghchi and Alderson, 1983) and 10 to 15 min (Barghchi and Alderson, 1985), respectively. In contrast, mature material is difficult to disinfect and high levels of contamination have been observed in nodal explants (Barghchi and Martinelli, 1984). The use of meristem-tip explants has been found to reduce contamination (Barghchi, 1985a). Establishment of up to two-year-old material was low at the first stages of culture initiation mainly because of severe browning. Improvements have been made by transferring the explants to new sites on the medium within the same vessel once browning was observed (Barghchi, 1985b). No benefit of reducing browning was obtained either from incubating cultures in the dark for up to one month (Barghchi and Alderson, 1985), or from pre-washing explants in distilled water for up to 60 min prior to culture (Barghchi, 1985b). Growing up to four-year-old mother plants in the glasshouse, severely pruning them and frequently subculturing the cultured explants has been reported to give good results (Martinelli, 1988). In the case of

explants taken from mature, fruit bearing trees, severe browning has been observed in Stage I and establishment has been difficult. Pre-soaking of explants in malonic acid ($10-100 \text{ mg l}^{-1}$) for up to 20 min and its supplementation at 50 mg l^{-1} to the culture medium only delayed browning marginally (Barghchi, 1985a). Omitting ferrous sulfate from the medium during the first subcultures almost eliminated browning (Barghchi, 1985a). Incubation in the presence of 2 mg l^{-1} BAP resulted in more browning of explants and media than in the case of 0.3 mg l^{-1} (Barghchi, 1985). More suitable material for subculture appeared to be provided by conditioned mother plants, i.e. pruned, repeatedly grafted, sprayed with BAP and GA_3 or from micrografting (Barghchi and Martinelli, 1984; Barghchi, 1985a). However, poor establishment was observed when explants of mother plants sprayed with GA_3 were used, and maintenance of shoots which resulted from in vitro micrografting meristem-tips onto juvenile rootstocks was difficult (Barghchi, 1985a).

Shoot-tips and nodal bud segments are the main explant types used for culture establishment (Barghchi and Alderson, 1983; Barghchi, 1985a; Barghchi and Alderson, 1985; Pontikis, 1985). Meristem-tips have also been investigated in the case of mature trees (Barghchi and Martinelli, 1984; Barghchi, 1985a). A strong apical dominance and better establishment and growth in culture were observed in shoot-tip cultures as compared with nodal bud segments. However, this difference was lost following subculturing on suitable media (Barghchi, 1985b; Barghchi and Alderson, 1985).

2.1.3.2 STAGE II : Shoot Multiplication

Micropropagation of Pistacia species is mainly achieved by inducing growth and development of multiple shoots from pre-existing apical and axillary buds. Murashige and Skoog (1962), or a modification of this medium supplemented with plant growth regulators have been used for shoot initiation and proliferation, respectively, for P. vera (Barghchi and Alderson, 1983; Barghchi and Martinelli, 1984; Barghchi and Alderson, 1985) and P. integerrima, P. atlantica and P. vera (Martinelli, 1988). In the case of P. terebinthus, macro- and micronutrients of Anderson (1978) formula were used (Pontikis, 1985). Multiple shoot proliferation was better with BAP than with kinetin, and

4 mg l^{-1} BAP was optimal for shoot proliferation of P. vera seedling material (Barghchi and Alderson, 1983). Lower BAP concentrations have been used by Martinelli (1988) to propagate one- to four-year-old P. vera plant material. Lower levels of BAP have also been used in the case of four- month to four-year-old P. atlantica (0.7 mg l^{-1}), P. integerrima (1 mg l^{-1}) seedling rootstocks and mature P. terebinthus trees (2.5 mg l^{-1}) (Pontikis, 1985). Following subculturing on media containing 4 mg l^{-1} BAP up to 40 and 35 shoots were obtained per culture respectively, in the case of seedling material (Barghchi and Alderson, 1983) and one- to two-year-old plants (Barghchi and Alderson, 1985). The addition of as little as 0.05 mg l^{-1} NAA to the culture medium inhibited seedling explant growth (Barghchi and Alderson, 1985) and higher concentrations increased callus growth at the base of P. vera seedling shoots (Barghchi and Alderson, 1983). Shoot growth and proliferation of seedling material was not improved by adding 0.25-4 mg l^{-1} GA₃ or GA₄₊₇ to the culture medium containing 4 mg l^{-1} BAP (Barghchi and Alderson, 1983).

Shoot-tip necrosis (STN) of cultured explants of pistachio during micropropagation has been reported to be a common problem (Barghchi and Alderson, 1985; Pontikis, 1985; Martinelli, 1988). Adding as low as 0.25 mg l^{-1} NAA to the medium containing 4 mg l^{-1} BAP increased the rate of shoots exhibiting STN from 13 to 42%, whereas no such symptoms were observed either when kinetin was used with or without NAA, or when shoots developed roots (Barghchi and Alderson, 1985). Barghchi and Alderson (1989) suggested that deficiencies of boron or calcium are most likely to be responsible for STN symptoms in pistachio. Martinelli (person. comm., 1989) was of the opinion that it is physical rather than chemical factors that are most likely to induce STN in vitro.

Induction of adventitious shoots and organogenesis in callus have also been investigated (Barghchi and Alderson, 1989). In the former case, adventitious shoots have been obtained from the mid-rib area as well as from around the margins of leaves attached to the shoots and in close contact with the medium; this has been accompanied with a quick or delayed senescence, respectively. In the latter case, root formation occurred mainly on callus originating from cotyledonary tissues and

shoot organization has been possible from callus obtained from nodal bud explants of P. vera (cv. Ohadi).

2.1.3.3 STAGE III : Rooting

Rooting has been achieved either in vitro (Barghchi and Alderson, 1983, 1985; Pontikis, 1985; Martinelli, 1988) or in vivo (Martinelli, 1988). IBA was found to be the most suitable auxin for in vitro rooting (Barghchi and Alderson, 1983, 1985). Rooting was improved by using half-strength macronutrients during the root induction stage, by incubating cultures under dark conditions for the first seven days of culture on root initiation medium and finally by transferring shoots to an auxin-free medium after the first roots reached 1 to 2 mm length (Barghchi and Alderson, 1983, 1985). No significant differences in rooting were observed with either 2.5, 3.0 or 3.5 mg l⁻¹ IBA, and 80% rooting with three roots per shoot was obtained with up to two-year-old P. vera seedling-derived material (Barghchi and Alderson, 1985). Poorer rooting was obtained when explants were taken from decapitated mature P. terebinthus (60%) (Pontikis, 1985), and from up to four-year-old grafted P. vera plants (30-40%) (Martinelli, 1988). In the former case, the rooting medium was supplemented with 1 mg l⁻¹ IBA and 89 mg l⁻¹ phloroglucinol.

2.1.3.4 STAGE IV : Weaning

Rooting of P. integerrima microcuttings taken from up to four-year-old seedlings was achieved in vivo after a quick dip in 100 mg l⁻¹ IBA and a good establishment of weaned plants was achieved within three weeks (Martinelli, 1988). In vitro rooted shoots were successfully established (70-80%) in peat-based compost in spite of the dark colour of roots. One root per explant was sufficient to ensure establishment (Barghchi and Alderson, 1985). By contrast, other experiments in which in vitro rooted P. vera shoots have been investigated, weaned plantlets failed to grow after being transplanted into a glasshouse (Martinelli, 1988). Artificial inoculation of in vitro rooted P. integerrima explants with vesicular-arbuscular mycorrhizae fungi was also investigated. Significant increases in plant growth of inoculated plantlets were obtained when unamended substrates were used (Schubert and Martinelli, 1988).

2.2 MICROGRAFTING

2.2.1 Introduction

Grafting consists of uniting two pieces of living plant tissue : a scion stem-piece (a piece of detached shoot containing one or several buds) and a rootstock (a rooted cutting, a layered plant or a seedling). It is an age-old technique; it was known to the Chinese at least as early as 1000 B.C. (Hartmann and Kester, 1983). Grafting has retained its interest because of the influence of the rootstock upon the scion and vice versa, i.e. the mutual interaction of the two genotypes (Tubbs, 1973), such as in the case of tree size and form, disease resistance and precocity of flowering. Moreover, grafting offers other advantages such as the propagation of difficult-to-root species and the ability to change cultivars within an already established orchard. In 1821, 119 traditional techniques of grafting had been already described (Thouin, 1821 in Hartmann and Kester, 1983), and in the mid 1920's a so-called "Micrografting" technique was investigated for the first time. This relatively new technique was an accommodation of the well-known grafting concept and procedures except that it could only be practiced on a small scale and required exceptional manual dexterity.

Micrografting is achieved either in vivo or in vitro. In vivo micrografting consists of grafting a very small scion originating from a desirable mother plant onto a young rootstock obtained either from a seedling or from an herbaceous cutting grown in a nursery or under greenhouse conditions. On the other hand, in the case of in vitro micrografting the scion is sterilized before use and the rootstock is either a seedling obtained from aseptically germinated seed or a micropropagated microcutting; the latter is used in order to overcome the problem of heterogeneity of some seedling rootstocks (such as in Fig) (Baud, 1981 in Jonard, 1986) and sweet cherries (Lacrotte, 1981 in Jonard, 1986). In vitro propagated shoots have also been used as rootstocks in peach tree (Negueroles and Jones, 1979) and in other Prunus species (Cornaggia, 1986). The scions used in both cases

consisted of meristem tips (0.1-0.4 mm) or apices (0.2-2.0 mm) (Jonard, 1986). Buds have also been reported to be used once they have been rid of their outlying scales (0.5-1.5 cm) (Jonard, 1986).

2.2.2 Micrografting Technique and its Applications

2.2.2.1 In vivo micrografting

Micrografting is used for different purposes. It was worked first in vivo in order to solve propagation problems and more particularly to produce virus-free plants. Because of difficulties in rooting virus-free shoots of Dahlia obtained from in vitro shoot-tip culture, in vitro obtained shoots were grafted onto virus-free seedlings and the first virus-free plants were therefore obtained from infected mother plants (Morel and Martin, 1952 in George and Sherrington, 1984). The same technique has also been used to recover virus-free chrysanthemum plants from infected ones by grafting 4-8 mm scions onto young plants (Holmes, 1956 in Jonard, 1986). Invariably, in the mid 1950's, the in vivo micrografting technique was exploited in order to rejuvenate mature ivy plants (Hedera helix) by grafting apical meristems onto young rootstocks (Doorenbos, 1953 in Jonard, 1986). Rejuvenation of mature forest trees by carrying out several regraftings has also been reported (Franclet, 1977). Recently, the in vivo micrografting technique has been used to propagate compact grafted nursery trees (Maurice, 1987). This method was recommended for distant locations because of the light weight and compact root configuration of the produced plants.

Besides the above reported species, fruit trees such as Citrus (Tusa et al., 1987) and Prunus (Colin and Verhoyen, 1976; Jonard et al., 1983) have been investigated. This technique has mainly been used in order to overcome the delicate stage of ex vitro establishment and, therefore, to obtain high numbers of virus-free plants.

2.2.2.2 In vitro micrografting

The in vitro micrografting technique was developed for the first time in 1971 by Murashige et al., (1972) who succeeded in micrografting shoot apices of several Citrus species and hybrids onto disease-free

seedling rootstocks. The main objectives of their work were the production of virus-free plants because of failures in in vitro shoot apex culture, and the avoidance of the typical juvenile phase of plants obtained from nucellus tissue.

In vitro micrografting has since been used by other workers for different purposes. These are considered as follows:

a) Virus elimination

The effectiveness of in vitro micrografting in producing virus and virus disease-like clean plant material has been investigated by several workers. An estimated 72.5% and 57% of peach tree plants were freed of sharka and of Necrotic Ring Spot Virus (NRSV), respectively, using 0.1-0.5 mm long apices (Jonard et al., 1983). Larger shoot tips (0.5-1.0 mm) have also been used to obtain virus-free peach trees: 70% of plants were freed of sharka (Mosella et al., 1979), while NRSV was totally eliminated (Cornaggia, 1986). Several strains of Prunus Ring Spot Virus (PRSV), Prune Dwarf Virus (PDV), and Chlorotic Leaf Spot Virus (CLSV) were eliminated from peach trees also by using 0.5-1.0 mm long shoot tips (Navarro et al., 1982). Using this approach, virus elimination was reported also in other fruit species, e.g., apple and cherry (Huang and Millican, 1980; Cornaggia, 1986; Deogratias et al., 1986a). The results obtained with peach were virus-type dependent : 100% of micrografted plants were free of CLSV strains which are clostero viruses, whereas the rates were highly variable (from 0 to 92%) in the case of PRSV and PDV (Navarro et al., 1982). The above obtained results and those of other workers investigating the elimination of tristeza and sharka seem to indicate that filamentous viruses are relatively easily eliminated by shoot tip grafting. Easiness with which elimination of viruses and virus-like diseases using shoot-tip grafting is achieved has been proven. For example, in the case of Citrus diseases these have been classified into three groups : very easy, easy and difficult to eliminate (Navarro, 1984).

Virus-free Citrus plants were obtained for the first time by Murashige and co-workers in 1971 when they were able to free some Temple tangor and Eureka lemon budlings of exocortis by grafting in vitro stem apices

with 4-6 leaf primordia excised from mature-bearing tree shoots onto disease-free seedling rootstocks (Murashige et al., 1972). A few years later, several Citrus cultivars had been apparently freed of psorosis and tristeza viruses, exocortis viroid and stubborn spiroplasma (Navarro et al., 1975). Since then, shoot-tip micrografting has been used to free Citrus selections from virus and virus-like diseases on a routine basis (Navarro et al., 1976; Roistacher et al., 1976; Roistacher and Kitto, 1977; Navarro et al., 1980, 1981; Starrantino and Carusu, 1987). Because of the heavy infection of Citrus trees in Spain with several virus and virus-like diseases, and considering the overall potential advantages of in vitro shoot tip grafting, this technique has been selected to be the pillar of the Citrus Variety Improvement Program in Spain (CVIPs) which started in 1975 (Navarro, 1976; Navarro et al., 1981). The outcome of this program showed that almost 100% of the micrografted plants have been freed of tristeza, exocortis, xyloporosis and vein enation. Only 65% of the tested plants have been freed of the difficult to eliminate pathogens producing psorosis-like young leaf symptoms (PLP) (Navarro et al., 1981). By 1984, over three million plants were obtained using shoot-tip grafting and delivered to growers in Spain; other countries have also been reported carrying out similar programs with a view to produce virus-free Citrus plants (Navarro, 1984).

Recently, numerous Citrus clones have been recovered from all the known virus diseases they carried, using apical meristem with 2 or 3 leaf primordia taken from clones raised under glasshouse conditions (Starrantino and Carusu, 1987). The beneficial effect of high temperatures under which shoot tip mother plants were grown on producing virus-free plants was reported in the case of four virus strains of peach tree (Navarro et al., 1982), and in the case of difficult to eliminate Citrus diseases (Navarro et al., 1980, 1981; Navarro, 1984).

The scion sources employed also affected levels of virus elimination achieved (Deogratias et al., 1986a). The virus-free plant rates obtained were 0, 40-70 and 100% for cherry apices excised from herbaceous shoots, dormant buds and in vitro cultured shoots, respectively. However, 20% of the potted plants appeared to be

contaminated after one month of their transfer to soil.

b) "Rejuvenation" and/or "Invigoration" of mature plant material

The ability to form adventitious roots has been reported to progressively change with the transition from the juvenile to the mature phase (Schaffalitzky, 1975 and Burger, 1980 in Hackett, 1985), and morphological and developmental changes associated with this transition are species dependent (Hackett, 1985). Woody perennials, in particular, are difficult to root in the adult phase (Sax, 1962; Heybroek and Visser, 1976).

Besides using juvenile parts of mature plants, rejuvenation of mature parts of plants using different techniques to restore rooting ability of cuttings taken from adult woody perennials has considerable practical implications. For instance, grafting mature scions onto juvenile rootstocks is being used to obtain relatively juvenile plant material in woody perennials such as Eucalyptus (Franclet, 1979), Citrus (Monselise, 1973) and avocado (Haas, 1937 in Pliego-Alfaro and Murashige, 1987).

Complete rejuvenation of previously mature scions (based on morphological and physiological characteristics) was reported on ivy (Hedera helix). However, because of the lack of supplementary information or experiments in many of the reported works, it has been difficult when a mature scion is grafted onto juvenile rootstock to relate the observed changes to reversal of physiological aging (invigoration) or to rejuvenation (Hackett, 1985).

The level of the graft, the size of the scion, the number of sequences of grafting and the presence of leaves have all been reported to influence the rooting potential of mature material when grafted onto juvenile rootstock. As a general rule, the more that the level of the graft approaches the rootstock's rooting system the greater the rejuvenation response that is obtained (Chaperon, 1979 in Hackett, 1985). Concerning scion size, small mature scions resulted in the best levels of rejuvenation obtained in Hedera (Stoutemyer and Britt, 1961), Eucalyptus (Franclet, 1979) and giant sequoias (Monteuuis, 1986 in Bon

and Monteuis, 1987). Scions with leaves inhibited rejuvenation; whereas, the rejuvenation of Hedera was enhanced when leaves were present on the rootstock (Doorenbos, 1954 in Hackett, 1985).

The effects on rooting of repeated grafting in more or less close intervals has also been investigated. Five successive regraftings with Hevea brasiliensis (Muzik and Cruzada, 1958 in Jonard, 1986) and three every two months with Eucalyptus camaldulensis (Franclet, 1979) were necessary to improve rooting potential of mature scions. In the latter case, six and twelve month intervals between regraftings did not enhance rooting competence.

Most of the information reported above has been derived from investigations using in vivo micrografting technique. In vitro micrografting has also been investigated in Citrus (Navarro et al., 1975), avocado (Pliego-Alfaro and Murashige, 1987), Pistachio (Barghchi and Martinelli, 1984; Barghchi, 1985a) and giant sequoias (Bon and Monteuis, 1987). In the first case, in vitro micrografted mature Citrus shoot tips grafted onto seedling rootstocks did not revert to the juvenile state and flowered 11 months after grafting (Navarro et al., 1975). With avocado, lateral buds taken from greenhouse-grown mature trees have been micrografted onto four-week-old seedlings and the resulting shoots tested for their rooting competence (Pliego-Alfaro and Murashige, 1987). Results indicated that 30% of the shoots rooted and the average number of roots was 1.9 per shoot. After being recut and retested twice, this poor rooting potential of the micrografted shoots was retained. Rooting percentage was increased up to 50% after the IBA concentration in the rooting medium was reduced. By contrast, 100% rooting was obtained in the case of juvenile shoots with a mean of 3.5 roots per shoot. Ungrafted mature material failed to root. Rooting competence was not improved after three successive regraftings of mature shoots onto seedling rootstocks. However, it was suggested that rooting might be improved with additional regrafts (Pliego-Alfaro and Murashige, 1987). It has, therefore, been inferred that the rooting competence restoration of mature-grafted shoots was due rather to reversion from mature to juvenile phase than to a transfer of rooting cofactors from juvenile rootstocks to mature scions (Pliego-Alfaro and Murashige, 1987).

In order to improve growth and proliferation of mature pistachio trees, in vitro micrografting of mature scions onto juvenile rootstocks was investigated. Results indicated that the growth of the scion was very slow and the cultures were difficult to maintain in vitro (Barghchi, 1985a).

Recently, a micro-2D PAGE electrophoresis technique has been used to study tree maturation and the rootstock's rejuvenation effect (Bon and Monteuis, 1987). One-hundred-year-old clones of Sequoiadendron giganteum were rejuvenated after mature meristems were micrografted in vitro onto young seedling rootstocks. Abundant protein populations, in particular acidic proteins, were observed in mature scions compared to the morphologically rejuvenated ones showing that proteic modifications within meristem tips accompanied scion morphological changes after micrografting.

c) Compatibility and incompatibility studies

In order to understand compatibility and incompatibility phenomena with a view to improve grafting success and to detect incompatibility at an early stage, in vitro micrografting has been investigated using different approaches.

Prunus graft formation studies showed that all the fundamental tissues contributed to the graft callus formation which occurred 48 hours after grafting (Deloire, 1982 in Jonard, 1986), and that cellular events and the sequence of events preceding graft union formation were similar to those observed for shield-budded trees and Prunus trees, respectively (Gebhardt and Goldbach, 1988). However, more vascular connections resulting from wound vessel members formation across the entire pith zone was observed in micrografted Prunus, and vascular cambium initiation was not found to be a condition of vascular connections establishment (Gebhardt and Goldbach, 1988).

Mitotic activity of both scion and rootstock was weak in the incompatible canino/apricot/myrobolan combination (Deloire, 1982 in Jonard, 1986), or even often non-existent in the case of the

exocortis-induced incompatibility in the lemon/Troyer citrange association (Martinez, 1979 and Monpouya, 1983 in Jonard, 1986).

Micrografting has also been explored as a way of rapidly detecting incompatibility at an early stage (Martinez et al., 1979; Deloire et al., 1983; Jonard et al., 1983; Chimot-Schall et al., 1986; Damiano et al., 1987). The apricot/myrobolan localized incompatibility was observed as early as the 14th day in culture and no micrografted plant survived by the 60th day. On the other hand, 30% of peach/apricot and 16% of peach/myrobolan translocated incompatible associations have been transferred ex vitro and survived plants represented 6% and 1%, respectively, by the 60th day (Martinez et al., 1981; Jonard et al., 1983). It has, therefore, been possible to detect incompatibility in less than two months instead of one to five years usually needed in the orchard in the case of translocated incompatibility (Tabuenca, 1967 and Herrero, 1968 in Martinez et al., 1981), and some 10 years after grafting in the case of localized incompatibility (Rogers and Beakbane, 1957 in Martinez et al., 1981). In lemon/Troyer citrange association, 10 to 15 days are sufficient to detect the exocortis induced incompatibility (Jonard, 1986) which would need some 20 years in the orchard (Fraser and Lewitt, 1978).

d) Other applications

Other applications of in vitro micrografting techniques such as the research into latent virus effects on cellular metabolism (Burger, 1984) and the study of rootstock influences on scion growth and development (Negueroles and Jones, 1979) have been suggested.

Recently, it has been reported that the in vitro growth following different combinations of Prunus micrografts paralleled ones obtained in the field. It has therefore been suggested that the obtained vigour in vitro could possibly be used to select dwarfing rootstocks (Gebhardt and Goldbach, 1988).

2.2.3 Practices and Problems

2.2.3.1 Factors affecting grafting success

Several factors have been reported to influence micrografting success during all the stages of the operation:

a) Factors associated with the rootstock

For Citrus species, seed germination is carried out in the dark on an agar-solidified medium since it has been found that light influenced grafting success. When seeds were germinated under 1,000 Lx illumination only 2.7% successful grafts were obtained in contrast to etiolated rootstocks which gave 37.7% success rate (Navarro et al., 1975). In the case of Rosaceae, seeds are first stratified under continuous darkness at 3-5°C for 30-120 days depending on species after a 24 to 48 hour imbibition period in sterile distilled water. Stratification is carried out in general in tubes on agar solidified medium (Alskief and Gautheret, 1977; Martinez et al., 1979; Huang and Millican, 1980). This long period of cold treatment has been drastically reduced to 24 hours for certain species by simply dipping seeds in cytokinin, gibberellic acid or a mixture of the two solutions (Rouskas et al., 1980).

The success rate is age- and species-dependent. The best results have been obtained with the dark-grown two-week-old Troyer Citrange rootstock (Navarro et al., 1975) and only with three-day-old Poncirus trifoliata but grown under light (Hosoi et al., 1979 in Navarro, 1984). When younger Citrus rootstocks were used, an early development of callus which overwhelmed shoot tips was observed, whereas drying and browning leading to death of most scions characterized the micrografting of older stocks (Navarro et al., 1975; Navarro, 1984). Similar observations have also been made for Prunus (Martinez et al., 1979).

Rootstocks with morphological markers have been preferred to others in order to ease the identification of the origin of new developing shoots after micrografting has been carried out. Marker rootstocks with

trifoliated leaves and with pigmented leaves (anthocyanin), respectively, have been respectively used for Citrus (Murashige et al., 1972; Navarro et al., 1975; Edriss and Burger, 1984) and Malus (Alskief and Villemur, 1978).

The cotyledons and root tips have not been found to influence grafting success and consequently their elimination has been recommended leaving 1.5 cm long epicotyls and 4-6 cm long roots without cotyledons or axillary buds (Navarro et al., 1975; Navarro, 1984).

b) Factors associated with the scion

Grafting success has been found to be positively correlated with the scion size (Navarro et al., 1975; Huang and Millican, 1980; Deogratias et al., 1986b). The successful grafts obtained ranged from 1.8-47% in Citrus (Navarro et al., 1975), and from 15-90% in Malus (Huang and Millican, 1980) when the scion size increased from 0.05-0.8 mm by including more leaf primordia. In the case of Prunus, no successful grafts were obtained with scions of less than 0.4 mm and the results increased from 15-65% when shoot tips of 0.4-1.0 mm were used (Deogratias et al., 1986b). However, increasing the scion size increases risks of virus contamination (Murashige et al., 1972; Navarro et al., 1975; Burger, 1984; Deogratias et al., 1986b). Consequently, shoot tips consisting of apical meristems plus three leaf primordia measuring 0.14-0.18 mm are most commonly used in Citrus (Navarro et al., 1975).

Pretreatment of apices before grafting has been reported to improve grafting success (Mosella et al., 1979; Jonard et al., 1983; 1987). A liquid medium supplemented with 0.1 mg l^{-1} of zeatin resulted in 64% successful peach tree grafts after a 48-hour pretreatment period instead of 22% obtained with the untreated control (Jonard et al., 1983).

Pretreatment for five days under complete darkness and for two days under $5,000 \text{ ergs.cm}^{-1}.\text{s}^{-1}$ with a 16-hour photoperiod was also shown to be beneficial for graft take (Jonard et al., 1987).

Considering the source of shoot-tips, the highest success rates have been obtained with flushes (30-40%) in contrast to freshly excised axillary buds (15-25%) harvested from the field or from glasshouse grown Citrus trees. Intermediate results (20-30%) have been obtained with flushes of in vitro cultured axillary buds. The latest method has the overall advantage of being season-independent (Navarro et al., 1975). Different results have, however, been obtained with cherry and peach cultivars (Deogratias et al., 1986a, b). Grafting success did not exceed 20% for shoot-tips taken from dormant buds of adult tree shoots harvested in January and stored at low temperature until their use in April to May, and were very low (3%) for cherry or even zero for peach cultivars when scions were obtained from the current season's growth and from buds of young shoots. The levels of take have been increased to 50% using juvenile material obtained from glasshouse-grown seedlings (Deogratias et al., 1986a, b). Improved rates (30-40%) approaching those obtained with juvenile material have been obtained using in vitro cultured shoots of cherry cultivars suggesting a possible rejuvenation of in vitro cultured material (Deogratias et al., 1986a). This hypothesis has been supported by the fact that a quicker scion growth was obtained using five or seven times subcultured material compared with the three times subcultured one.

Grafting success is also influenced by the time of the year at which shoot tips are used (Mosella et al., 1979; Huang and Millican, 1980; Poessel et al., 1980). The outcome of a detailed experiment conducted during an apple tree growing season showed that grafting success was very low (10%) from November through March, but increased up to 70% in May, the month after which the success rate decreased by about 10% per month until October and then was stabilized around 10% (Huang and Millican, 1980). Other periods such as September (Mosella et al., 1979) and June (Poessel et al., 1980) have been reported optimal for peach tree micrografting. This season-dependent response was overcome by using shoot-tips excised from in vitro cultured shoots and the grafting take reached 60% (Huang and Millican, 1980). Biochemical studies of apices excised from mature springtime peach tree cultivars showed that their phenolic content did not seem to affect success in spite of high levels of accumulation in apices from March through July. However, an inverse correlation (a highly significant

coefficient of correlation of -0.7946) was obtained between grafting success and peroxidase activity. This activity was weak in June, corresponding to the best period for grafting (Poessel et al., 1980), and this could be due to its limiting effect of browning or to its interactive regulation of endogenous auxin (Poessel et al., 1980; Jonard, 1986).

c) Factors associated with the grafting procedure

Several grafting procedures have been attempted. The placement of shoot-tips onto rootstocks was found to influence grafting success (Navarro et al., 1975; Edriss and Burger, 1984; Cornaggia, 1986; Jonard et al., 1987). Placing shoot-tips either on the vascular ring tissue at the top cut-surface of the decapitated Citrus epicotyl, or on the cortical surface within the inverted "T" incision made at the top of the decapitated rootstock gave the highest success rates (Navarro et al., 1975; Navarro, 1984). The overall levels of successful grafts were between 5 and 50% but no significant difference existed between the two mentioned methods which resulted in 50 and 45% grafting success, respectively (Navarro et al., 1975). Both methods have also been reported equally effective with 40-50% establishment in peach (Negueroles and Jones, 1979). The second method resulted, however, in the case of grapefruit in grafting successes ranging from 27.7 to 46.6% depending on the rootstock used as compared with 15.5-16.9% for the first method (Edriss and Burger, 1984). In pistachio, placing mature shoot-tips (with few leaf primordia) at the top of the cut surface of juvenile rootstocks was better than inserting scions in a vertical incision made on the decapitated rootstocks (Barghchi, 1985a).

Recently, another method consisting of performing a lateral micrograft without decapitating the rootstock and which was inspired from the chip-budding technique was developed for apple, peach and cherry cultivars. The results obtained exceeded 50%, whereas the success rate did not exceed 5% when apices were placed at the top cut surface of the decapitated rootstock (Cornaggia, 1986).

Pretreatments of shoot tips and/or rootstocks have also been investigated to improve graft establishment (Alskief and Gautheret,

1977; Martinez et al., 1979; Mosella et al., 1979; Jonard et al., 1983; Starrantino and Carusu, 1987). Pretreatments consisted of dipping plant material in growth regulators before grafting, applying external growth regulators and/or antioxidants after grafting, or inserting a block of agar containing growth regulators, nutrients and/or antioxidants. Grafting success of grapefruit was doubled after dipping shoot tips before grafting for 5-10 minutes in 10 mg l^{-1} 2,4-D or in 1 mg l^{-1} kinetin. The average successful grafts increased from 30.0 to 74.4 and 75.7%, respectively (Edriss and Burger, 1984). Dipping both Citrus shoot-tips and the decapitated seedling rootstocks for 10 minutes in 0.5 mg l^{-1} BAP increased the success rate from 63 to 91% (Starrantino and Carusu, 1987). In peach, external application of cytokinin as droplets on the cut surface of the rootstock has also been reported (Mosella, 1979). Treatment with zeatin ($0.01\text{--}1.00 \text{ mg l}^{-1}$) increased the success rate from 21.7 to 54.5%; the highest results being obtained with 1 mg l^{-1} of zeatin when a vermiculite support was used. Grafting success was also increased by using BAP at 0.1 mg l^{-1} , but the rate did not exceed 39% (Mosella et al., 1979). Better results have been obtained by combining apices pretreatment before grafting and the external application of cytokinin at the graft union. Thus, 64% of the grafts were established when shoot tips were pretreated with 0.1 mg l^{-1} zeatin for 4, 24 or 48 hours, and when 0.1 mg l^{-1} of zeatin was applied to the cut surface of rootstocks at the time of grafting (Mosella et al., 1979).

In order to prevent scion desiccation and to stimulate histogenesis, the insertion of a small agar block between the scion and the rootstock has been considered one possibility (Alskief and Gautheret, 1977; Martinez et al., 1979; Jonard et al., 1987; Pliego-Alfaro and Murashige, 1987). Grafting success was considerably increased up to 85% in the case of Citrus by use of a 0.6% agar block containing MS mineral nutrients, vitamins, sucrose (75 mg l^{-1}), BAP (1 mg l^{-1}), NAA (0.5 mg l^{-1}) and GA₃ (1 mg l^{-1}) (Jonard et al., 1987). Improvements have also been reported in the case of apricot and peach scions when they were grafted onto apricot rootstocks using agar block containing in addition to MS mineral nutrients, vitamins, sugar (40 mg l^{-1}) and IBA and GA₃ at 1 mg l^{-1} . However, this technique did not seem to increase the success rate when apricot scions were grafted onto peach seedlings

(Martinez et al., 1979), and no increase was observed in the case of different peach cultivars when micrografted onto the GF305 rootstock (Alskief and Gautheret, 1977).

Concerning the oxidation occurring at the cut surfaces of shoot tips and rootstocks which was reported to prevent a good graft establishment, antioxidant substances were used for rinsing shoots, during shoot-tip excision or in droplets placed onto the cut surface of the rootstock just before performing the graft. Sodium diethyl-dithiocarbamate (DIECA) at 2 mg l^{-1} is commonly used (Martinez et al., 1979; Mosella et al., 1979; Jonard et al., 1983; Cornaggia, 1986; Deogratias et al., 1986b).

d) Factors associated with the culture of the micrografts

Liquid medium combined with a filter bridge perforated in its centre is routinely used to establish micrografted plants in vitro. However, liquid medium has been reported to cause root asphyxiation and necrosis (Mosella et al., 1979; Deogratias et al., 1986a). Several substrates and supports have, therefore, been tested. Results of such investigations showed that a synthetic support called 'Milcap', made from polypropylene fibres and humidified with liquid medium resulted in better graft development when compared with perlite or vermiculite supports. Moreover, this support has the advantage of easing micrografting manipulations, promoting root development, allowing a harmonious development of the rooting system and facilitating the transfer to soil of developed micrografts (Deogratias et al., 1986a). The obtained graft take were 50-60% for juvenile cherry material, and 20% for scions excised from mature dormant buds grafted onto seedling rootstocks; this compared to 5.0 and 0.5%, respectively, when a liquid medium was used. Agar-solidified medium has recently been used to culture avocado micrografts and 70% successful grafts have been obtained (Pliego-Alfaro and Murashige, 1987).

Addition of growth regulators has been proven to be an effective method for increasing success rates in peach. Response was better with IAA than with NAA and IBA, and grafting success was increased from 9 to 48% just by adding as little as 0.3 mg l^{-1} IAA in the liquid medium used to

feed the micrografts (Mosella et al., 1979). In contrast, no improvement was obtained with IAA or with BAP ($0-10 \text{ mg l}^{-1}$) in Citrus (Navarro et al., 1975). In Pistacia, supplementation of 0.3 mg l^{-1} BAP and $0-0.05 \text{ mg l}^{-1}$ IBA to the culture medium was found to be more suitable than the optimal level of BAP used for shoot multiplication, i.e. 4 mg l^{-1} (Barghchi, 1985a). When pretreatment of apices with zeatin (0.1 mg l^{-1}) for 4, 24 or 48 hours was combined with adding zeatin (0.1 mg l^{-1}) to the liquid medium (with vermiculite), it was possible to obtain 64% successful grafts (Mosella et al., 1979). In Citrus, 75% takes were obtained when shoot-tips were pretreated with NAA ($2.6 \text{ } \mu\text{M}$) and BAP ($3.3 \text{ } \mu\text{M}$) (added to the medium), and a 100% success rate was achieved by combining the above cited treatments with insertion of an agar block containing phytohormones between shoot-tips and rootstocks (Jonard et al., 1987).

The sucrose content of the medium stimulated leaf growth, new lateral root production and affected significantly micrografting success. In Citrus species, increasing sucrose concentration up to 75 mg l^{-1} resulted in 90% successful grafts as compared with 55% obtained with 25 and 50 mg l^{-1} , respectively (Navarro et al., 1975). Similar results (95%) have also been obtained with high sucrose concentration (85 mg l^{-1}) instead of 40% resulting from using $30-50 \text{ mg l}^{-1}$ (Monpouya, 1983 in Jonard, 1986). In contrast, lower concentrations (15 to 45 mg l^{-1}) were preferred in the case of apple (Alskief and Villemur, 1978). The highest results (29%) were obtained with 30 mg l^{-1} of sucrose, and no successful graft was obtained either with 60 or with 75 mg l^{-1} .

Light appears also to play an important role (Navarro et al., 1975; Alskief and Gautheret, 1977; Deogratias et al., 1986b; Jonard et al., 1987). When micrografted plants were kept under continuous darkness, no successful graft was obtained (Navarro et al., 1975), and callus production was reduced (Deogratias et al., 1986b). By contrast, high light intensity resulted in apex necrosis (Deogratias et al., 1986b). Illumination level of $3,000 \text{ lx}$ was found optimal in the case of apple cultivars when compared with 400 and $1,100 \text{ lx}$ (Alskief and Villemur, 1978), and a quantum flux density of $5 \text{ } \mu\text{E.s}^{-1}.\text{m}^{-2}$ better than 10 or $50 \text{ } \mu\text{E.s}^{-1}.\text{m}^{-2}$ in the case of cherry and peach cultivars (Deogratias

et al., 1986b). In contrast, 16-hour daily exposure to light illumination ranging from 300 lx to 10,000 lx did not seem to be critical in Citrus and, therefore, 1,000 lx was retained as standard (Navarro et al., 1975). A 14-day pretreatment period under darkness before micrografts were exposed to $5,000 \text{ ergs. cm}^{-2}.\text{s}^{-1}$ with a 16-hour photoperiod was effective for improving success rates in Citrus (Jonard et al., 1987).

2.2.3.2 Transfer and acclimatization of micrografted plants

Three to eight weeks after micrografting, established plants are usually transferred to soil (Navarro et al., 1975; Jonard et al., 1987; Gebhardt and Goldbach, 1988). An additional two-week period under high light intensity (10,000 lx) was recommended for Citrus before potting micrografted plants in soil (Navarro, 1984).

Different substrates such as peat:sand:perlite (2:1:1) (Gebhardt and Goldbach, 1988), peat:sand (1:1) (Jonard et al., 1987) have been used during this stage, and the obtained survival rates have exceeded 60% (Navarro et al., 1975; Navarro et al., 1982 ; Navarro, 1984; Gebhardt and Goldbach, 1988).

The substrate used to support micrografts before their transfer to soil has also been found to be critical for ex vitro establishment (Negueroles and Jones, 1979; Cornaggia, 1986; Deogratias et al., 1986a). The 'Milcap' support allowed 60-70% survival rate in cherry because of the good development of the root system, and because the plantlets were transferred with their supports without being damaged (Deogratias et al., 1986a). Almost 100% survival was obtained with apple, peach and cherry using this system in contrast to micrografts previously cultured on agar-solidified medium for which delayed growth and aleatory results were observed (Cornaggia, 1986).

2.2.3.3 Field evaluation of in vitro micrografted plants

Shoot-tip graft budlings of 'Willow leaf' mandarin and 'Temple' tangor obtained through in vitro micrografting, and freed of tristeza virus and exocortis and cachexia viroids were budded on 'Troyer' citrange

rootstocks and planted in 1976 for evaluation. The trees obtained were apparently true-to-type and no obvious genetic changes appeared to have taken place. Other observations on 'Temple' tangor, 'Washington' navel orange, 'Clementine' mandarin and 'Bears' lime confirmed also the apparent uniformity of in vitro micrografted materials (Nauer et al, 1983).

CHAPTER THREE

GENERAL MATERIALS AND METHODS

CHAPTER THREE : GENERAL MATERIALS AND METHODS

3.1 PLANT MATERIAL

Nuts of Pistacia vera were harvested from trees of the 'Mateur' cultivar grown at Ain Taoujdat research station (INRA) in the Meknes area, and those of Pistacia atlantica were obtained from wild species growing in Khemisset area. Both regions are located in the northern part of Morocco. After blank seeds of P. atlantica were eliminated following water soaking on flotation, the hulls (pericarp and mesocarp) were removed and the nuts stored dry inside a polythene bag kept in the dark at 4°C. Seedling explants were obtained from seeds germinated and grown under in vitro conditions. Mature material was obtained from four-year-old P. vera cv. Mateur plants grown in 15-l plastic pots or from thirty-year-old trees located in an orchard on the Ain Taoujdat research station. In the former case, plants were kept in a shaded area under glasshouse conditions and their leaves were removed to force axillary buds to sprout.

3.2 MEDIA PREPARATION

Murashige and Skoog (1962) medium, as supplied by 'Flow Laboratories' in powder form, was used throughout this study except in some experiments where stock solutions of Murashige and Skoog (1962), Anderson (1978), Knop (1865) in George and Sherrington (1984) and Lloyds and McCown (1981) media were prepared individually from 'Analar' grade chemicals supplied by 'B.D.H.' Ltd and 'Sigma'. In the latter cases, stock solutions of macronutrients (x10), micronutrients (x100) and vitamins (x100) were stored at 4°C (for nutrients) and at -20°C for vitamins. Plant growth regulators, supplied by 'Sigma', were also prepared as stock solutions (0.1 mgml⁻¹) and were stored at 8°C. Sucrose (30 gl⁻¹) was added to the media at the time of preparation and double deionised water (DDW) was used for all the preparations. In the case of agar-solidified media, all components were dissolved in half the final volume of DDW and the pH adjusted to 5.7-5.8 using either 0.5M or 0.1M NaOH or HCl, and agar (Technical Grade 3, Oxoid) at 7 gl⁻¹ was dissolved by heating to 80°C separately in the second remaining

half volume of DDW. Equal volumes of nutrients and agar preparations were successively dispensed into the culture vessels with final volumes of 10 and 26 ml for tubes (75 x 25 mm) and 100 ml jars, respectively. In the case of liquid media, a supportive platform was made from a folded cellulose chromatographic paper and placed in 10 ml of nutrient solution in tubes (filter paper bridge system). The platform was perforated in its centre for insertion of the root portion of the rootstock used in micrografts. By contrast, seeds were placed directly on top of the paper platform. The culture vessels were covered with aluminium oxoid caps or 'Magenta' plastic lids and media autoclaving was carried out at 121°C under a pressure of 100 KPa for 20 min. Dissecting instruments were sterilized in an oven at 200°C for 120 min. Before use, they were immersed in 70% ethanol and then dipped for a few seconds in a 'Steribead 250' dry-heat sterilizer maintained at a 250°C setting.

3.3 EXPLANT PREPARATION AND CULTURE INCUBATION

The hard endocarps of P. vera and P. atlantica seeds were removed manually and chemically (1N H₂SO₄), respectively. Surface sterilization of explants was carried out (unless otherwise stated) using 20% (v/v) sodium hypochlorite solution ('Jeyes Brobat' bleach at Wye and 'Chlorox' bleach in Morocco) supplemented with 0.1% Tween 20 as a wetting agent using a shaker (Gallenkamp) or a vacuum infiltration system and the process was completed with three rinses of 2 min each with DDW. Shoots were excised into 15-20 mm long explants each containing 3-4 axillary buds and cultured on agar-solidified medium. The culture vessels were covered with sterile polypropylene sheets held in place with rubber bands and were incubated in a growth room at 25°C/16h photoperiod (standard conditions) unless otherwise stated. Fluorescent lamps (Philips TLD, 50W) were used to provide irradiance at culture tube level of 55-62 $\mu\text{Em}^{-2}\text{s}^{-1}$.

3.4 GLASSHOUSE CONDITIONS

In vitro rooted shoots and germinated seeds were gently washed briefly under running water and immersed for 1-2 s in a mixture of Captan and Benlate at 1 mg l^{-1} before they were potted in a mixture of peat and

grit (1:1, v:v). Weaning was achieved in two stages in MacPenny (UK, Ltd) fogging units: the first was maintained at 90±5% R.H. (for 3-4 weeks) and the second at 70±5% R.H. (for 2-3 weeks) before plantlets were transferred to normal glasshouse conditions (65±5% R.H.). The fogging unit was operated at 23°C (bottom heat) in ambient range of 15-20°C. Nutrient feed (0.5 mg l⁻¹ of Libfeed 311; 31:10:10) was provided to plants undergoing weaning on a regular weekly basis.

3.5 LIGHT MICROSCOPY (LM) STUDIES

LM studies were conducted to investigate the graft union development of in vitro micrografts of P. vera using a modified method of Johansen (1940). Sections of 5-10 mm long specimens which had been cut at the graft union level were used throughout this investigation and the procedure of preparation was as follows:

Stage	Compounds	Time(h)
1. Fixation	Formol-acetic-alcohol	24
	- Formalin	5%
	- Glacial acetic acid	5%
	- Absolute ethanol	60%
	- Distilled water	30%
2. Dehydration	Distilled water:Ethanol:	
	Methylpropanol	(1:1:1) 24
		(5:4:1) 24
		(3:5:2) 24
		(1:5:4) 24
		(0:4:6) 24
		(0:2:8) 24
		(0:0:1) 24(x3) ^a
3. Infiltration	Paraffin wax and Methylpropanol (60%)	24
	Paraffin wax (Fibrowax)	24(x3)
4. Wax Embedding		

^a 0.5 g l⁻¹ erythrosin, a temporary stain, was added during the first change to ease locating specimens.

Longitudinal sections of 7 µm thickness were cut using a rotary microtome (Bright Instrument Company) and were floated on glass slides on top of which a drop of Haupt's adhesive (brushed over) and of distilled water were added. The slides were left overnight on a warm

slide dryer, wax was removed using 'Histo-clear', and the preparations hydrated. The sections were stained in 1% safranin (made up in 50% ethanol) for one hour and counterstained in 0.5% fast green (made up in 90% ethanol) for a few seconds before they were dehydrated, cleaned and mounted in 1-2 drops of Histomount. The slides were allowed to harden on a warm slide drier before they were examined under an inverted microscope (Olympus IMT-2).

3.6 FLUORESCENT MICROSCOPY (FM) STUDIES

In order to investigate the graft union formation, and in order to ease and speed the procedure usually used (i.e., LM), FM was attempted. Fresh longitudinal hand sections of 20-30 μm thickness were made at the graft level of in vitro micrografts of P. vera and stained in 0.01% w/v aqueous acridine orange to reveal nuclei. Sections were mounted in DDW for examination under U.V. inverted microscope (Olympus IMT-2).

3.7 ELECTRON MICROSCOPY (EM) STUDIES

3.7.1 Low Temperature Scanning Electron Microscopy (SEM)

Leaves of in vitro shoots of P. vera were rapidly dissected into small fragments of about 5 mm^2 and attached to a metal 'stub' and frozen in liquid nitrogen at -196°C . After gold coating for 3 mins at about -186°C , specimens were observed at -160 to -180°C using a low temperature SEM system (Hexlamb Cryotrans 1000) attached to the SEM (Hitachi S430) in order to investigate the functioning of stomata at different environmental conditions in stage II cultures.

3.7.2 Transmission Electron Microscopy (TEM)

This technique was used to investigate the cell ultrastructure of shoots affected by shoot-tip necrosis (STN), as well as the necrotic layer at the graft union level following in vitro grafting. Specimens of small size (0.5 mm^3) were used in these studies and their preparation was as follows:

Stage	Compounds	Time
1. Fixation	2.5 % Gluteraldehyde in 0.1 M cacodylate buffer (CAB) pH7	12 h
	0.1 M CAB pH7	10 min(x2)
	5.0mM O_3O_4 in 0.1 M CAB pH7	2 h
	0.1 M CAB pH7	10 min(x2)
2. Dehydration	50, 70, 80 and 90% acetone	10 min(x4)
	100% acetone (stored over desiccant)	30 min
3. Embedding	Acetone : Resin (2:1)	20 min
	(1:1)	20 min
	(1:2)	20 min
	Resin (Epon-araldite)	12 h
	Resin	12 h
4. Polymerization	Resin (60°C oven)	48 h

The obtained blocks of hard resin containing the specimens were first trimmed using a stereo-microscope and then 0.5 μ m thick sections were cut with a glass knife mounted on an ultra-microtome (Reichert-Jung Ultracut E) and finally stained in 1% Toluidine blue (made up in 1% Borax) for LM examination. Once the area of interest had been selected silver-gold ultra-thin sections of 70 nm thickness were cut using a diamond knife and were mounted on grids of copper mesh covered with Formvar plastic. The mounted sections were stained first in 5% uranyl acetate (made in 1% acetic acid) for 30-45 min, and then carefully washed in DDW. After staining for 5-10 min in Reynolds lead citrate, the grids were washed in DDW and allowed to dry at room temperature before being examined under TEM (AEI/Kratos Corinth 500).

3.8 DATA COLLECTION

Recordings of contamination rate, browning of culture media, necrosis of explants and survival rates of explants were made during Stage I of culture. In the case of seeds, the germination levels were also taken. For assessing shoot proliferation during Stage II, shoot numbers, total shoot lengths and total bud numbers were recorded after four weeks of culture. The ultrastructures of cells of necrosed shoot-tips and leaves were examined by EM. During stage III, rooting levels and numbers of roots per microshoot were recorded. At Stage IV,

survival rate data were collected. In the case of micrografting, success rates, degrees of callus formation at the graft unions, levels of axillary shoot development and elongation increments of scions were recorded. Graft union formation was examined under an inverted microscope (LM and FM).

3.9 EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Treatments in all statistically analysed experiments were arranged in a completely randomised design. ANOVA was carried out and the least significant difference (LSD) at $P=0.05$ probability level was used to separate the means of different treatments. In the case of proportional data, the Chi-square (χ^2) test was used whenever appropriate. The significance levels used were as follows:

- $P>0.05$ = not significant
- $P<0.05$ = significant
- $P<0.01$ = highly significant
- $P<0.001$ = very highly significant.

CHAPTER FOUR

MICROPROPAGATION OF PISTACHIO

CHAPTER FOUR : MICROPROPAGATION OF PISTACHIO

4.1 STAGE I : CULTURE ESTABLISHMENT

In the initial stage in the micropropagation process, establishment of P. vera and P. atlantica seeds as well as of P. vera explants have been investigated. In the latter case, shoots and/or buds of four- and 30-year-old grafted plants were used.

4.1.1 STAGE IA : Establishment of shoot cultures from seed, bud and shoot materials

4.1.1.1 Establishment of P. vera and P. atlantica seeds

a) Effects of conditions of storage and method of sterilization on asepsis of P. vera seeds

Surface sterilization of P. vera seeds has been reported by Barghchi and Alderson (1983) to be performed firstly in 70% ethanol for 45 s and secondly in 20% (v/v) sodium hypochlorite solution for 25 min. However, data on the effectiveness of the above-cited method have not been published. In the present experiments, therefore, the effects of seed storage conditions and the method employed for surface sterilization were studied.

Samples of seeds of P. vera either stored under ambient conditions or maintained dry at 4°C were surface sterilized as described in Section 3.3. Surface sterilization was carried out for 20 min using either a shaker, or a vacuum infiltration system. The effects of a pretreatment of seeds with 70% ethanol for 45 s were also studied in the case of this material treated by shaking.

In the cases where surface sterilization was carried out using a shaker, all seeds stored under ambient conditions were infected in contrast to those stored previously at 4°C. In the latter case, only 5% of the cultures were infected (Table 1). In both cases no benefit was gained from pretreating seeds with 70% ethanol. In the case of

those batches of seeds stored dry at 4°C, surface sterilization using the vacuum method appeared to reduce slightly the levels of contamination from 5.0 to 1.7%; 100 and 66.7% of the observed contaminations were of bacterial origins in the case of vacuum- and shaker-treated seed batches, respectively.

TABLE 1 : Effects of conditions of storage and method of sterilization on asepsis of P. vera seeds (n=60) (% contamination)

Condition of storage	Shaker		Vacuum	χ^2_{test} (2 df)
	+ Ethanol	- Ethanol	- Ethanol	
Ambient	100.0	100.0	n.d. ^a	(P>0.05)
Dry (4°C)	5.0	5.0	1.7	(P>0.05)
χ^2_{test}	(P<0.001)	(P<0.001)		

^a n.d. = not determined.

b) Effects of method of sterilization on asepsis of P. atlantica seeds

In preliminary experiments, seeds of P. atlantica seemed to become sensitive to high concentrations of sodium hypochlorite and were extremely fragile during surface sterilization. In the present experiment, the effects of using reduced levels of bleach and the use of the less mechanically disruptive vacuum system were investigated.

After the hard endocarps were chemically digested using H₂SO₄, and carefully removed using thin forceps (Section 4.1.1.2,b), the seeds were surface sterilized (Section 3.3) using a shaker or a vacuum. In the former case, the effects of diluting bleach solution to 12% and surface sterilizing seeds twice for 10 min each were also investigated.

The obtained results (Table 2) showed that in the case of the shaker treatment, infections increased from 10.8% up to 54.1% when the concentration of bleach and the time of surface sterilization were reduced even though the sterilization process was repeated twice. Disinfestation by vacuum gave a similar result to the one obtained with the shaker when 20% of bleach was used. It is to be noticed here that

no fungal contamination was observed in the case of vacuum-treated seeds and all the infections observed were of bacterial origins. In the case of shaker-treated seeds, 13.5 and 2.7% fungal contaminations were observed when 12 and 20% bleach were used, respectively.

TABLE 2 : Effects of method of sterilization on asepsis of P. atlantica seeds (n=37)

	Shaker		Vacuum	χ^2_{test} (2 df)
	12% Bleach (x2)	20% Bleach (x1)	20% Bleach	
% Infected cultures	54.1	10.8	10.8	(P<0.001)

c) Effects of medium strength and GA₃ on germination and seedling growth of P. vera

Preliminary experiments showed that agar-solidified medium was not suitable for germinating seeds of P. vera due to the development of intense browning of the media during culture which necessitated at least two successive subcultures onto fresh media before this problem was overcome. Barghchi and Alderson (1983) germinated P. vera seeds on filter paper platforms in 60 ml jars containing 2-3 ml of liquid medium. In the subsequent studies on seed germination, the filter paper bridge system and liquid media were adopted. In the present experiment, the effects of media strength and GA₃ on germination and seedling growth were investigated.

Shelled P. vera seeds were germinated in tubes using DDW as well as different strengths of MS medium ($\times \frac{1}{10}$, $\times \frac{1}{4}$ and $\times \frac{1}{2}$). GA₃ effects were also tested using its absence or presence at 10 mg l⁻¹. Cultures were incubated for 12 days under standard conditions.

Germination levels ranged from 63.6 to 68.2% when different strengths of MS medium were used (Table 3). The presence of GA₃ at 10 mg l⁻¹ in $\times \frac{1}{2}$ MS media increased germination up to 81.8% as compared with 68.2% in its absence, but this increase was not significant. Concerning seedling growth, increasing the medium strength resulted in an improvement of shoot length and bud number with a significant effect in

TABLE 3 : Effects of medium strength and GA₃ on germination and seedling growth of P. vera
after 12 days of culture (n=22)

	DDW	MS strength				F _{test} (45 df)	LSD (0.05)	X ² _{test} (4 df)
		1/10	1/4	1/2	1/2 ^a			
Shoot length (mm)	25.3	30.0	35.0	39.0	42.5	(P<0.01)	10.32	
Bud number	6.5	6.8	7.7	7.9	8.1	(P>0.05)	1.26	
Root length (mm)	80.0	64.0	111.7	88.0	84.3	(P>0.05)	39.29	
% Germination	63.6	68.2	68.2	68.2	81.8			(P>0.05)

^a Medium supplemented with 10 mg l⁻¹ GA₃.

the former case. There was also an increase (without being significant) of shoot length due to the supplementation of $1/2 \times$ MS with 10 mg l^{-1} GA₃. Root length did not seem to respond significantly either to the medium strength or to GA₃.

d) Effects of medium strength on germination and seedling growth of P. atlantica

Preliminary experiments showed that blank seeds represented 20% of the total number of seeds tested and that some form of scarification was necessary before germinating P. atlantica seeds in vitro. To remove the hard endocarp, seeds were soaked in H₂SO₄ and were gently shaken. The appropriate time for chemical scarification was 90 min; 60 min was not enough and 120 min damaged either partially or totally both the embryos and cotyledons. Consequently, soaking P. atlantica seeds in water to distinguish blank seeds and a chemical scarification treatment of 90 min duration were adopted. Two subsequent experiments were designed to investigate firstly, the effects of medium strength on germination and seedling growth of P. atlantica and secondly, the effect of storage duration on germination of seeds.

Unlike P. vera seeds, the use of solidified medium was possible without a need for subculturing P. atlantica seeds because of the very low level of browning of media. Scarified seeds of P. atlantica were germinated in tubes using either DDW or a range of MS medium strengths ($\times 1/10$, $\times 1/4$, $\times 1/2$ and $\times 1$). The media were solidified with 7 mg l^{-1} agar and the cultures were incubated for 13/20 days under standard conditions.

Germination increased from 47.4 up to 100% when seeds were germinated on increasing strengths of MS medium (Table 4). A significant increase in germination was observed in batches of seeds germinated on full strength MS medium as compared with all the other treatments. Concerning shoot growth, increasing the strength of the medium significantly affected shoot and root lengths but not bud numbers. In all cases, full strength MS medium was optimal.

TABLE 4 : Effects of medium strength on germination and seedling growth of P. atlantica after
13 days of culture (n=20)

	MS strength					F _{test} (39 df)	LSD (0.05)	X ² _{test} (4 df)
	DDW	1/10	1/4	1/2	Full			
Shoot length (mm)	20.3	22.7	29.3	29.7	36.5	(P<0.05)	10.64	
Bud number	4.2	4.2	4.2	4.2	4.2	(P>0.05)	0.42	
Root length (mm)	59.8	74.7	82.2	77.5	105.1	(P<0.05)	30.09	
% Germination	47.4	50.0	50.0	55.5	100.0			(P<0.01)

e) Effects of storage duration on germination of P. atlantica seeds

It was reported that seeds of Pistacia species are unlikely to be effectively stored (IBPGR, 1986 in Barghchi and Alderson, 1989). In the present investigation, germination of P. atlantica seeds was followed over different durations of storage at low temperature.

Germination of scarified P. atlantica seeds was tested at harvest and after 1, 2, 3 and 12 months of storage. Seeds, kept in-shell in plastic bags, were stored dry at 4°C. Germination was assessed after three weeks of incubation under standard conditions.

At harvest, 100% of the seeds germinated in vitro. However, a significant reduction of germination level of P. atlantica seeds was detected following two months of storage. This became negligible (1.3%) after 12 months of storage (Figure 1).

4.1.1.2 Establishment of four-year-old P. vera derived shoots

Browning of media and explants has generally been attributed to the oxidation of phenolics. It is a common problem limiting in vitro establishment of species containing or exuding high levels of phenolic compounds especially in the case of woody species (Hu and Wang, 1983; Thorpe and Patel, 1984; Zimmerman, 1985). Several approaches have been used to reduce this problem; these have included the use of antioxidants, the incubation of cultures under reduced light or in total darkness and the frequent subculture of explants to fresh medium during culture initiation (Hu and Wang, 1983).

In the following experiments, the use of antioxidants as well as the effects of duration of dark pretreatment combined with handling procedures on establishment of four-year-old P. vera explants were investigated.

a) Selection of suitable antibrowning agents

In order to control the release of tannins and phenols by mature material of several nut tree species, various antioxidants such as

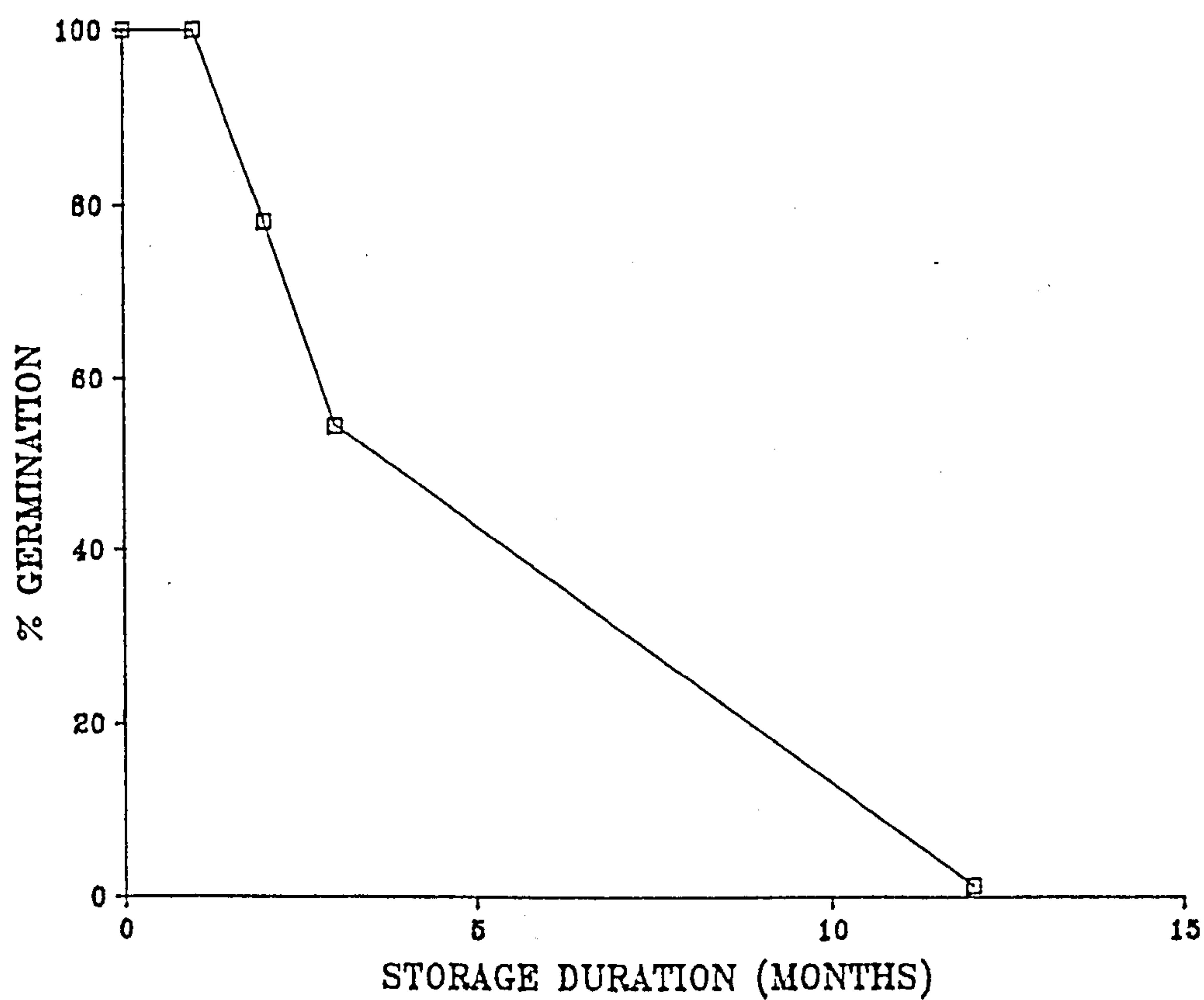


FIGURE 1 : Effects of storage duration on germination in vitro of P. atlantica seeds (n=50-117).
 χ^2_{test} (4 df) : ($P < 0.001$).

citric acid and ascorbic acid (Vieitez and Vieitez, 1983; Rodriguez et al., 1989a) and adsorbent agents such as Polyvinylpyrrolidone (PVP) and activated charcoal (Chevre et al., 1983) were used.

In pistachio, presoaking seedling explants of up to two-year-old plants and supplementation of media with 50 mg l^{-1} malonic acid did not prevent browning and only marginally delayed it (Barghchi, 1985a). Martinelli (person. comm. 1989) also found no benefit or even negative results from using antioxidants or charcoal.

In the present experiment, the effects of ascorbic acid, citric acid, PVP and charcoal on preventing media browning were investigated.

Leaf discs of 9 mm diameter were excised from surface sterilized leaves of four-year-old P. vera plants and were cultured in a natural orientation on agar-solidified MS medium in petri-dishes. Media were supplemented with 4 mg l^{-1} BAP, 10 mg l^{-1} PVP, 100 mg l^{-1} citric acid (CA), 100 mg l^{-1} ascorbic acid (AA), 100 mg l^{-1} CA + 100 mg l^{-1} AA and 3 mg l^{-1} activated charcoal (CC). AA and CA were filter sterilized and were supplemented to the media before dispensing. Cultures were incubated under standard conditions and diameters of the area of media which became brown were measured and relative intensities of browning of the media were visually assessed after one week of incubation.

The obtained results (Table 5) showed that AA completely prevented browning of culture media and its effect was significantly different from all the combinations tested. In contrast, neither CA nor PVP reduced browning; the worst results were obtained with the former compound. Although CA in combination with AA as a mixture reduced browning, this was significantly different from the result obtained with AA alone. Supplementation of CC did not prevent browning and the presence of BAP did seem to increase browning although not to a significant extent.

b) Effects of antioxidants and the method of sterilization used

After AA was found efficient in preventing browning of the culture medium when leaf discs were used, effects of its supplementation to the medium in combination with the method of sterilization used were investigated.

TABLE 5 : Size and intensity of media browning following incubation of P. vera leaf discs in the presence of a range of anti-browning agents (n=8)

	Control	BAP (4 mg l ⁻¹)	Antibrowning agents ^a				Ftest (42 df)	LSD (0.05)	
			PVP	CA	CA+AA	AA			CC
Diameter of brown area (mm)	22.3	23.0	23.4	24.6	19.4	9.0	nd	(P<0.001)	2.11
Intensity of browning ^b	++	++	++	++	+	-	nd		

nd : not determined because of difficulties in assessing browning of the medium.

- ^a Antibrowning agents:
PVP = polyvinylpyrrolidone (soluble) (10 mg l⁻¹)
CA = citric acid (100 mg l⁻¹)
AA = ascorbic acid (100 mg l⁻¹)
CC = activated charcoal

^b Intensity of browning:
- no browning
+ faint browning
++ intense browning

Explants of four-year-old P. vera were surface sterilized using either the shaker or vacuum infiltration methods and were cultured on solidified $1/2 \times$ MS medium containing 0.5 mg l^{-1} BAP with or without AA at 100 mg l^{-1} . Cultures were incubated in the darkness at 25°C . Browning of media and explants as well as the establishment and contamination levels were assessed after 3, 7 and/or 21 days of incubation.

The results presented in Table 6 showed that in all cases browning was observed first on culture media at three days after inoculation while browning of explants did not appear until after one week of culture. By three weeks, AA reduced browning of the media but did not reduce browning of explants. Browning of explants was increased substantially when the surface sterilization method employed was vacuum infiltration. Survival of cultures after three weeks was high; 70 and 87.5% establishment in the case of vacuum- and shaker-sterilized materials, respectively, were obtained independently of the presence or absence of AA in the media. No contamination was observed in cultures produced from explants treated with either of the two methods of surface sterilization.

c) Effects of combined dark pretreatments and handling method used

Although biosynthesis of phenolic compounds is reported to be increased by light (Smith, 1973), no benefit was obtained from incubating pistachio cultures under dark conditions for up to one month at culture initiation (Barghchi and Alderson, 1985). Frequent subculturing of explants during the first stages of culture has been practised effectively in pistachio shoot culture to overcome media browning (Barghchi and Alderson, 1985; Martinelli, 1988).

In the present experiment, the effects of handling methods used in combination with durations of dark pretreatment applied during initiation of pistachio shoot culture were investigated.

Surface sterilized nodal explants of four-year-old P. vera were established on agar-solidified medium using the two following methods:

TABLE 6 : Effects of sterilization method (vacuum vs shaker) and supplementation of culture medium with AA on establishment of four-year-old P. vera explants after 3, 7 and 21 days of incubation

		% Browning of media			% Browning of explants			% Survival
Days		3	7	21	7	21	21	
Method	Ascorbic acid							
vacuum	-	0.0 ^a	30.0	70.0	0.0	50.0	70.0	
	+	0.0	10.0	30.0	0.0	25.0	70.0	
shaker	-	0.0 ^b	50.0	87.5	0.0	25.0	87.5	
	+	0.0	12.5	25.0	0.0	25.0	87.5	

a and b represent n=10 and 8, respectively.

Method 1 : Explants were first cultured on hormone-free medium and incubated in the dark at 25°C for three days and then subcultured on low level BAP-containing medium (0.1 mg l⁻¹) for a further three days before they were subcultured onto a shoot proliferation medium (MS medium supplemented with 2 mg l⁻¹ BAP). Cultures were finally incubated under standard conditions.

Method 2 : Explants were kept in darkness for three weeks (without any manipulations being carried out) on a low level BAP-containing medium (0.1 mg l⁻¹) before they were subcultured on the proliferation medium as specified above for the first method. Levels of media browning, explant necrosis, survival and callus development were assessed after three weeks of culture.

The results presented in Table 7 showed that incubating cultures for three weeks in darkness using Method 2 lowered the level of necrotic explants and resulted in a significant reduction in media browning as compared with subculturing them twice and keeping them only one week under dark (Method 1). Although callusing was observed in all cultures, high levels of callus production was noticeable when the first method was used. Long etiolated axillary shoots were produced when cultures were dark pretreated for three weeks and their elongation was significantly higher than that obtained with the first method. Establishment of explants was significantly affected by the method used for culture initiation with 50 and 93% survival being obtained using the first and second methods, respectively. The surviving explants were easily maintained thereafter on the shoot proliferation medium (Stage II).

4.1.1.3 Establishment of 30-year-old P. vera derived buds

Actively growing shoots have represented the main sources of plant material used for establishing several nut tree species in vitro (Chevre et al., 1983; Vieitez et al., 1983; Kester et al., 1986). Buds have also been used such as in the case of almond (Kester et al., 1986) and chestnut (Vieitez et al., 1986) but not in the case of pistachio. A series of experiments was carried out to investigate the establishment

TABLE 7 : Effects of method of culture initiation on establishment of four-year-old P. vera explants

	Medium browning		Necrotic explants		Survival (%)		Callus development		Shoot length increments ^c	
	(%)	Intensity ^d	(%)	(%)	(%)	(%)	(%)	size ^e	(mm)	
Method 1 ^a	100.0	++	27.8	50.0	100.0	+++	2.6			
Method 2 ^b	14.3	+	7.1	92.9	100.0	+	3.8			
X ² test (1 df)	(P<0.001)	(P>0.05)	(P<0.05)	(P>0.05)						
F test (30df)										(P<0.001)
LSD (0.05)										0.19

a and b Methods described in text (In each treatment n=18 and 14, respectively).

c Log_e transformed data.

Intensity of browning		Callus development	
+	Faint	+	Low
++	Intense	++	Medium
		+++	High

of 30-year-old Pistacia materials firstly using axillary buds (either nonforced or forced to leaf out) as bud culture systems, or larger explants (i.e., nodal and apical segments) as shoot culture systems.

a) Forced winter buds

Lignified shoots (formed in the previous spring) were collected from field-grown P. vera trees in December 1987 and were stored at 4°C until their use one month later. At that time the bases of the shoots were cut back and freshly cut ends then immersed in tap water contained in glass beakers held in a growth room at 25°C/16 h photoperiod in order to force the axillary buds to develop. Water was replaced and the cut ends of the shoots trimmed on a regular daily basis. The newly formed leafy shoots were excised and surface sterilized in 12% 'Chlorox' for 20 min using a shaker. The last of the three sterile rinses was performed with DDW supplemented with 100 mg l⁻¹ AA. Explants were cultured on agar-solidified MS medium in total darkness for one week before they were subcultured on fresh MS medium containing 4 mg l⁻¹ BAP and incubated under standard conditions. Levels of contamination, media browning and survival of shoots produced were assessed after three weeks following subculture.

Following this procedure, contamination was high (63%), all of which was of a fungal nature. Browning of the culture medium was a common problem being present in all of the cultures. Although 14.3% survival was achieved after three weeks following subculture, the further maintenance of established shoots proved difficult due to their slow growth and marked chlorosis.

b) Non-forced winter buds

In the previous experiment, a low concentration of 'Chlorox' was used to prevent damaging the fragile leafy shoots which were forced to develop from winter buds. Since this objective was not achieved in most cases, and because of the high levels of contamination obtained, non-forced buds were used in the present experiment. Axillary buds were excised from stored shoots (as specified in the previous experiment) and their hard scale-leaves removed. Surface sterilization

was completed as previously mentioned for forced buds except that the concentration of 'Chlorox' and the time of sterilization were increased up to 25% and 30 min, respectively. Buds were grown on liquid MS medium supplemented with 2 mg l^{-1} BAP and cultures were incubated in darkness at 25°C . Levels of contamination and survival were assessed after three weeks of culture at which stage the sprouting of buds had been initiated.

The results of this culture establishment approach demonstrated that there was an effective reduction in contamination levels (reduced to 30%), and establishment was improved with 20% sprouting shoots obtained after three weeks of culture. However, maintenance of these surviving explants was difficult as in the previous experiment. The remaining non-sprouted buds died.

c) Summer buds

Semi-hardwood shoots were harvested in mid-July 1988 and were stored at 4°C for one week. Surface sterilization was performed in 20% 'Chlorox' bleach solution for 20 min. The sterilized shoots were washed and treated with AA as previously described in (Section 4.1.1.3a). Axillary buds were then excised and cultured on MS liquid media either hormone-free or supplemented with 0.1, 0.5, 1, 2 or 4 mg l^{-1} BAP. Cultures were incubated for two days in total darkness at 25°C before being transferred into light conditions. Levels of contamination, bud browning and callus production were assessed after three weeks following incubation of shoot cultures in the light.

The results obtained (Table 8) showed that bud browning was 100% when the BAP level used in media exceeded 0.5 mg l^{-1} and that reductions in the levels of this cytokinin used in media reduced browning. Callus production occurred in all treatments with substantial increases in callus development on shoots when media were supplemented with BAP. Although no contamination was observed in these cultures, no effective establishment of rapidly proliferating shoot cultures could be obtained.

TABLE 8 : Effects of BAP on establishment of buds of 30-year-old P. vera trees after three weeks of culture (n=12)

	BAP (mg l ⁻¹)					
	0.0	0.1	0.5	1.0	2.0	4.0
% Bud browning	41.7	58.3	75.0	100.0	100.0	100.0
% Callus production	33.3	66.7	66.7	83.3	83.3	83.3

4.1.1.4 Establishment of 30-year-old P. vera derived shoots

a) Effects of different methods of surface sterilization

Actively growing shoots were harvested in May 1989 from 30-year-old trees and these were surface sterilized in one of the following ways:

Method 1 = Soaking in 24% 'Chlorox' for 20 min.

Method 2 = Soaking in 12% 'Chlorox' twice for 10 min each.

In both cases a shaker was used and 100 mg l⁻¹ AA was included in the last rinsing solution and the culture medium. Nodal segments were grown on hormone-free MS medium in total darkness and were subcultured twice during the first week and then again after two and four weeks of culture. The last subculture was carried out onto MS medium supplemented with 2 mg l⁻¹ BAP.

The results presented in Table 9 showed that contamination was relatively low (21-23%) following surface sterilization by either of the methods. When present in cultures, contamination was mainly fungal. A substantial increase in media browning was observed when shoots were surface sterilized twice (Method 2) and the browning levels were intense as compared with those present in cultures prepared using Method 1. Explants showed necrosis only when Method 2 was used and necrotic explants represented 18.8% after one week of culture. Following four weeks in culture, as much as 52.9 and 37.5% survival was achieved with explants prepared using Method 1 and 2, respectively. However, the surviving explants showed signs of vitrification and a slow decline in growth which was characterized by marked increase of shoot chlorosis.

TABLE 9 : Effects of surface sterilization method on establishment of actively growing (spring) shoots after four weeks in culture

Sterilization ^a method	Explant No.	Media browning ^b			Survival ^d (%)
		contamination (%)	Intensity ^e (%)	Necrosis ^c (%)	
1	34	20.6	11.8	0.0	52.9
2	48	22.9	41.7	18.8	37.5
χ^2 test (1 df)		(P>0.05)	(P<0.01)	(P<0.05)	(P>0.05)

^a Methods described in text
^b After two days in culture
^c After one week in culture
^d After four weeks in culture
^e Intensity of browning:
+ Faint
++ Intense

b) Effects of position of explants on mother plant

The origins of explants and in particular their position on mother plants have been reported to influence their establishment and growth in vitro (Vieitez et al., 1983; Barghchi and Alderson, 1985; Yu and Meredith, 1986). An experiment was carried out next to investigate whether terminal shoot-tips or nodal explants from mature P. vera were more responsive in culture.

Semi-hardwood shoots were harvested in mid-July 1989 and surface sterilized in 20% 'Chlorox' for 20 min using a shaker. Both terminal and basal sections of shoots were excised and cultured separately on MS medium supplemented with 0.1 mg l^{-1} BAP and cultures incubated for three weeks in total darkness before being transferred to standard light conditions. Subculturing was carried out as described in the previous experiment.

Browning was observed in all media in both terminal and basal explants with an intense browning in the former case (Table 10). The position of the explant influenced significantly the level of necrosis exhibited by explants with 71.4% necrotic terminal explants as compared with none

TABLE 10 : Effects of position of explants on mother plants on establishment of cultures from semi-hardwood shoots after three weeks of culture

Explant position	Explant No.	Media browning		Necrosis (%)	Survival (%)
		(%)	Intensity ^a		
Terminal	16	100.0	++	71.4	28.6
Basal	14	100.0	+	0.0	84.6
X^2_{test} (1 df)		(P>0.05)		(P<0.01)	(P<0.05)

^a see Table 9.

in the case of basal ones. A significant increase in surviving explants was obtained using basal as compared with terminal explants, i.e., 84.6 and 28.6%, respectively. No fungal contamination was observed and only one explant in each treatment showed bacterial infection. However, the maintenance of the surviving explants in vitro was again found difficult to achieve as in previous experiments.

4.1.2 STAGE IB : Multiple Shoot Formation (MSF) from Germinating Seeds

The initiation of multiple shoot cultures directly from germinating seeds has recently been investigated in several nut tree species such as in walnut (Rodriguez, 1982; Rodriguez et al., 1989a) and in hazelnut (Rodriguez et al., 1989b). This has been achieved by growing seeds on media containing BAP. The main advantage of this procedure is to speed up and increase proliferation of axillary shoots on seedling material. So far, no application of this technique has been reported for Pistacia seeds.

4.1.2.1 MSF from P. vera germinating seeds

Seeds of P. vera were germinated on $1/2$ x MS liquid media either hormone-free or supplemented with 2, 4, 6, 8 or 10 mg l^{-1} BAP and cultures incubated under standard conditions. Proportions of seeds with MSF, total shoot and bud numbers as well as levels of vitrification produced on seedlings were recorded after 14 and/or 35 days of culture.

Results showed that there was a substantial effect of BAP on MSF (Figure 2a) and the shoot and bud numbers per culture (Table 11) after 14 days of culture. Generally, these increased with the presence of raised levels of BAP in the media up to optimal levels being obtained at 8 mg l^{-1} BAP. Shooting responses did not differ significantly from the ones obtained at 6 mg l^{-1} BAP. However, a significant drop in MSF and the shoot and bud numbers was noticeable at 10 mg l^{-1} BAP. After 35 days of incubation, BAP at 0-8 mg l^{-1} stimulated MSF in all seeds as compared with a 90% response obtained at 10 mg l^{-1} . The number of shoots produced was higher but followed a similar trend as the one observed after 14 days of culture.

TABLE 11 : Effects of BAP on stimulation of shoot and bud formation in germinating P. vera seeds after 14 and/or 35 days of culture (n=10)

BAP (mg l ⁻¹)									
	Incubation period(days)							F _{test} (54 df)	LSD (0.05)
		0	2	4	6	8	10		
Number of shoots/seedling ^a	14	1.1	1.1	1.2	1.6	1.9	1.5	(P<0.001)	0.35
	35	2.0	2.2	2.4	2.7	2.9	2.7	(P<0.001)	0.35
Number of buds/seedling ^a	14	2.4	2.4	2.8	3.3	3.4	2.8	(P<0.001)	0.38

^a SQR transformed data.

Concerning vitrification (Figure 2b), BAP at levels above 4 mg l⁻¹ clearly increased vitrification levels (>55.6%) by 14 days. However, by 35 days, symptoms were observed in all cultures of all treatments.

4.1.2.2 MSF from P. atlantica germinating seeds

Seeds of P. atlantica were germinated on agar-solidified MS media either hormone-free or supplemented with 0.5, 1, 2, 4 or 8 mg l⁻¹ BAP. Cultures were incubated under standard conditions. Proportions of seeds with MSF as well as the total shoot and bud numbers forming on seedlings were recorded after 11 and 35 days of culture.

After 11 days of culture, no real differences were observed in MSF levels (Figure 3) and shoot and bud numbers (Table 12) in response to BAP levels in the media. Stimulation of MSF was observed only at 0.5 and 1 mg l⁻¹ BAP levels. After 35 days, it seemed that MSF was BAP-dependent in this species. BAP supplementation to the medium increased MSF levels up to 83.3%, the optimum level being obtained at 1 and 2 mg l⁻¹ BAP. However, a sharp reduction in the number of seeds stimulated to produce multiple shoots was observed when levels > 4 mg l⁻¹ BAP were supplemented to the culture media. A similar trend was observed for shoot and bud numbers with optima being obtained at 1 mg l⁻¹ BAP.

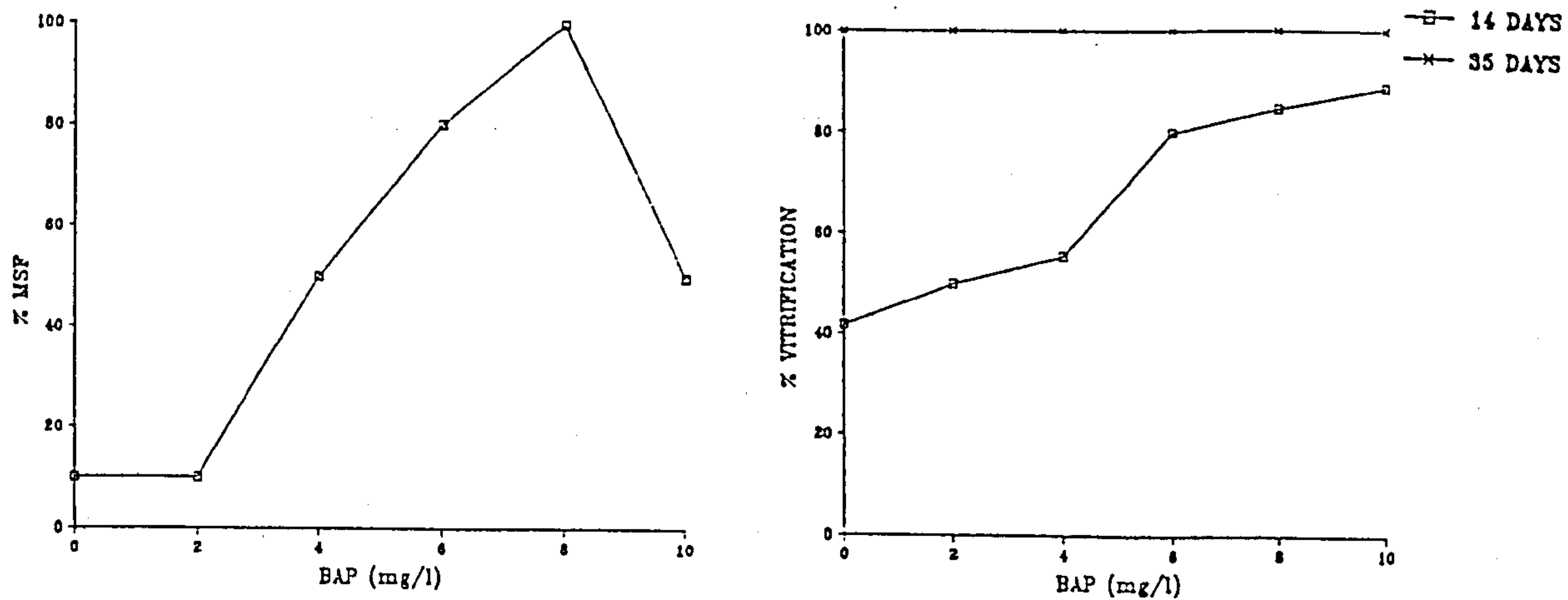


FIGURE 2a : Effects of BAP levels on MSF of germinating *P. vera* seeds after 14 days of culture (n=10).
 χ^2 test (5 df) : ($P < 0.001$).

FIGURE 2b : Effects of BAP levels on vitrification of *P. vera* seedlings after 14 and 35 days of culture (n=10).

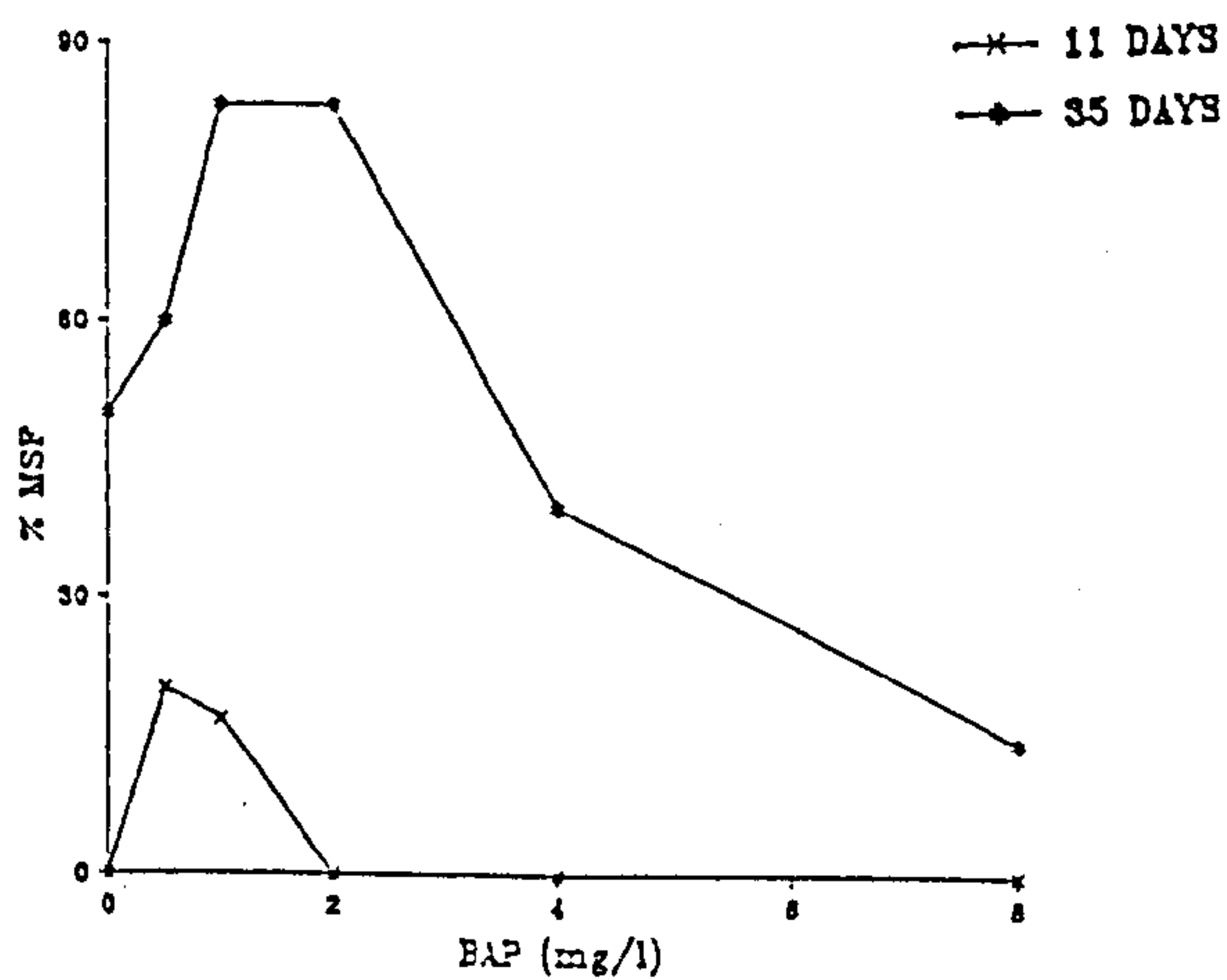


FIGURE 3 : Effects of BAP levels on MSF of germinating *P. atlantica* seeds after 11 and 35 days of culture (n=9).

TABLE 12 : Effects of BAP on stimulation of shoot and bud formation in germinating P. atlantica seeds after 11 and 35 days of culture (n=9)

BAP (mg l ⁻¹)									
Incubation								F _{test}	LSD
period(days)		0.0	0.5	1.0	2.0	4.0	8.0	(48 df)	(0.05)
Number of shoots/seedling	11	1.0	1.3	1.3	1.0	1.0	1.0		
	35	1.5	2.4	3.2	2.8	2.2	1.3	(P<0.05)	1.62
Number of buds/seedling	11	4.0	4.3	4.3	4.0	4.0	4.0		
	35	6.8	11.6	13.8	13.2	10.2	6.7	(P>0.05)	7.6

4.1.3 Discussion

Initiating cultures with clean and contamination-free plant material is important to the success of in vitro propagation. Selection of suitable explants and the use of appropriate methods of sterilization are essential requirements for successful shoot proliferation and growth in vitro (Mantell et al., 1985).

4.1.3.1 Establishment of P. vera and P. atlantica seeds

Investigations on surface sterilization of Pistacia seeds (Section 4.1.1.1a,b) showed that although the use of a vacuum system completely prevented fungal contamination, presumably by reducing the risk of trapping air bubbles at the surface of explants (Hu and Wang, 1983), complete disinfection was difficult to achieve, especially when the seeds were stored at ambient conditions. This was mainly because of bacterial infections. No benefit was gained either from pretreating seeds of P. vera with 70% ethanol for 45 s (Section 4.1.1.1a) or from surface sterilizing P. atlantica seeds twice for 10 min each using diluted bleach solution (12%) (Section 4.1.1.1b). Bacterial contaminations are reported to be possibly due to endogenous bacteria growing internally in the tissues (Hu and Wang, 1983; Zimmerman, 1985).

The production of empty (blank) nuts is a common problem of several if not all Pistacia species (Crane, 1973). In the present study, 20% seedless fruits were produced in the case of P. atlantica (Section 4.1.1.1d). Blank production in pistachio is reported to be mainly due to seed abortion (Bradley and Crane, 1975) as well as to parthenocarpy (Crane, 1973).

Acid scarification and GA₃ treatments are reported to be effective for overcoming seed dormancy (Hartmann and Kester, 1983). In the current work, acid scarification was necessary in the case of P. atlantica seeds to reduce the thickness of the hard and indehiscent endocarp, and to hasten germination (Section 4.1.1.1d). Soaking seeds for 90 min in sulphuric acid was optimal in producing seeds with very thin endocarps which were easily removable. A similar treatment followed by soaking seeds for 24 h in water was recommended for hard pistachio seeds before

their plantation (Joley, 1979, Shekafandeh and Shaybany, 1986). Once the endocarps were removed, seeds germinated easily without any pretreatment and 100% germination level was obtained at harvest. Most P. atlantica seeds are reported to germinate in vivo without pretreatment other than soaking in water for 1-2 h (Joley and Opitz, 1971) or for 24 h (Joley, 1979). Taghzouti (1984) found that soaking P. atlantica seeds for 24 h followed by stratification at 4°C for 30 days was beneficial in contrast with GA₃ pretreatment which reduced germination. Seeds of P. vera (cv. Mateur) also germinated without any pretreatment (Section 4.1.1.1c). Although supplementing the medium with 10 mg l⁻¹ GA₃ in the current study increased the germination of seeds, this improvement however, was not significant. Up to 95% germination was reported to be achieved in vivo following pretreatment of P. vera seeds with 50 mg l⁻¹ GA₃ for 24 h, and no benefit was gained from using levels up to 200 mg l⁻¹ (Taghzouti, 1984). In a recent publication, Frutos and Barone (1988) recommended 200 mg l⁻¹ GA₃ as a beneficial germination treatment for P. vera seeds. Taghzouti (1984) reported that differences in responses of P. vera seeds to GA₃ might be due to their different chilling requirements.

In the present investigation, storage was detrimental to seed germination; this became negligible after only one year from harvest (Section 4.1.1.1e). P. atlantica seeds were stored dry in plastic bags at 4°C in agreement with a commonly followed practice (Joley and Opitz, 1971). The obtained results confirm those of the IBPGR report of 1986 (in Barghchi and Alderson, 1989), stressing that Pistacia seeds are unlikely to be stored effectively. Other species such as Castanea crenata and Juglans nigra have also been reported to be recalcitrant (King and Roberts, 1979). Several factors have been reported to affect seed viability during storage such as certain aspects of production which affect seed viability at harvest, and the conditions of storage (mainly temperature and humidity) which influence the rate at which physiological changes take place (Hartmann and Kester, 1983; Bewley and Black, 1985; Mathews, 1988).

The use of a filter paper bridge system on which to germinate P. vera seeds offered a better possibility to solve the problem of browning of the culture medium which occurred when agar-solidified medium was

used. In practice, soaking P. vera seeds in frequent changes of water at 4°C for two weeks prior to planting was reported to be effective in stimulating rapid germination (Joley and Opitz, 1971). The purpose of leaching either by placing seeds in frequent changes of water or by soaking them in running water is reported to remove germination inhibitors (Hartmann and Kester, 1983). In the case of P. atlantica, these problems were not encountered because an extremely low level of browning of the medium usually occurred during germination.

The growth of P. vera in vitro was better and faster than that of P. atlantica (Section 4.1.1.1c,d). Supplementation of the culture medium with nutrients was beneficial in the case of both species in respect of seedling elongation, and in the case of P. vera for increasing the total bud number. In the case of P. atlantica, optimal germination of seeds was obtained when full strength MS medium was used. In the nursery, growth of P. atlantica seedlings has been reported to be slower than that of P. vera (Joley, 1979). However, when P. vera cultivars were top-worked on P. atlantica seedlings, they soon outgrow and outyield those on their own roots. Noticeably, long tap roots with very little or no side branching were obtained in the case of both species, and this resulted often in bending of roots in the bottom of the culture vessels. In the nursery, similar features have been reported in pistachio and pruning the long roots has been recommended to stimulate an even distribution of roots in containers (Richard et al., 1967 in Joley and Opitz, 1971).

Although P. vera and P. atlantica seedlings grew and developed well in vitro, shoot-tips soon became necrotic and died back. This phenomenon, referred to as shoot-tip necrosis (STN), was studied in detail in Section 4.2.4.

The results of the preliminary experiments on establishment of P. vera and P. atlantica shoots from seedlings showed the importance of using pistachio seeds which had been stored previously dry at low temperatures in order to avoid excessively high incidences of contamination. The use of a vacuum method to surface sterilize Pistacia seeds also seemed efficient in preventing fungal contaminations. Good germination and seedling growth could be obtained

using $1/2$ strength MS (liquid) medium in the case of P. vera, and full strength MS (semi-solidified) medium in the case of P. atlantica. In the latter case, scarification with sulphuric acid for 90 min was optimal and it proved essential in order to germinate seeds of this species in vitro.

4.1.3.2 Establishment of four- and 30-year-old P. vera-derived materials

Concerning the establishment of adult materials from four- or 30-year-old P. vera, complete decontamination of cultures was generally possible when either actively growing shoots were forced to develop from plants grown under protected glasshouse conditions and explants taken from such shoots (Section 4.1.1.2b) or, when buds were excised directly from semi-hardwood shoots of field-grown trees (Section 4.1.1.3c). Low levels of contamination were obtained with semi-hardwood shoots of field-grown trees, and intermediate results were obtained with actively growing shoots of field-grown trees (Section 4.1.1.4a) as well as with winter buds (Section 4.1.1.3b). Results showed, therefore, that in shoot culture the younger the explant is the easier it is to surface sterilize. They also showed that decontamination was more successful when glasshouse-grown mother plants were used as compared with field-grown trees. Difficulties in disinfecting mature as well as field-grown plant material have been reported for other woody species such as chestnut (Vieitez et al., 1983). It seems also from the results obtained in the current work that success in obtaining contamination-free explants (from field-grown trees) is season-dependent, in the case of both shoot and bud cultures. The best results were obtained with summer-harvested materials as compared with those harvested in spring (for shoots) (Section 4.1.1.4) or in winter (for buds) (Section 4.1.1.3). Less contamination has been reported previously to be obtained when explants have been harvested in mid-spring or summer than in autumn or winter (Hutchinson, 1982 in George and Sherrington, 1984).

Although media browning was observed in all cultures of terminal and basal sections of shoots obtained from 30-year-old P. vera trees, its intensity was higher when the former explants were used. Moreover, 71%

of terminal explants became necrotic and none in the other case (Section 4.1.1.4b). One hypothesis is that terminal sections might have contained higher endogenous levels of phenolic compounds such that they reached lethal levels once oxidized in wounded tissues. For instance, the phenolic content was found to be consistently and significantly higher in terminal as compared with axillary explants of grape-vine (Yu and Meredith, 1986). Moreover, a very high correlation was found in that work between the preexisting phenolic contents of the shoot-tips and the subsequent survival and production of shoots in culture. The fact that Barghchi and Alderson (1985) obtained the opposite results in the case of one-year-old P. vera, in which higher levels of establishment were obtained with shoot-tip explants as compared with nodal bud segments might have been due to the low phenolic content of explants of one-year-old plants as compared with the mature tree tissues (30-year-old) used in the current study. Actually, the same authors as well as others such as Martinelli (1988) reported that browning of tissues and media became more severe with increasing age of tissues. Alternatively, it might also be possible that terminal explants having been younger and softer than basal sections, could have been damaged more extensively during surface sterilization. In rose shoot cultures for instance, shoot-tips have been reported to be more sensitive to excess sodium hypochlorite than axillary buds, and the former type of explants have been severely injured once the sterilization treatment has been excessive or when the rinsing of explants has been incomplete (Khosh-Khui and Sink, 1982a in George and Sherrington, 1984).

The use of a vacuum system to surface sterilize pistachio explants caused more tissue browning and resulted in less survival than when a shaker system was used (Section 4.1.1.2b). The air removal taking place during the sterilization process when the vacuum was applied might have resulted in more tissue injuries and thus an increase in the subsequent liberation of phenolic compounds from tissues. When the concentration of the disinfecting agent and the time of application were reduced by half (but the process of surface sterilization repeated twice) more media browning was noticed, greater levels of necrosis of explants were observed and explant survival was markedly reduced (Section 4.1.1.4a). This suggested that the type of method used for

surface sterilization might simultaneously cause tissue damage or might even kill tissues as has been suggested and confirmed by several authors such as Hu and Wang (1983) and Vieitez et al., (1986), respectively.

Concerning the use of antibrowning treatments, no convincing results were obtained with the use of a range of previously exploited antioxidants and adsorbing agents. In the case of ascorbic acid, although encouraging results were initially obtained in the leaf disc plating assay (Section 4.1.1.2a), this treatment later proved ineffective for preventing browning in pistachio shoot cultures (Section 4.1.1.2b). The ineffectiveness of several antibrowning treatments which has been reported for other species such as chestnut (Chevre et al., 1983) and walnut (Rodriguez et al., 1989a) might partially be due to the fact that only solid media were being used as has been suggested by Ichihashi and Kako (1977) in Hu and Wang (1983).

In vitro establishment was possible from four- as well as thirty-year-old P. vera using actively growing shoots and/or buds, but this was more easily achieved in the former case. Maintaining the surviving explants was difficult in the case of explants taken from trees, since explants showed vitrification as well as a slow decline in growth with associated chlorosis (Section 4.1.1.2,3,4). Difficulties in establishing mature material, which has been reported to be a common problem facing the successful establishment of shoot culture of many woody species (Zimmerman, 1985), have been reported previously for pistachio (Barghchi and Martinelli, 1984; Barghchi, 1985a). Marco-Antonio (1984) even found that 10-year-old P. vera and P. atlantica were unresponsive to in vitro initiation. In the field, pistachio trees are known to produce fewer vegetative buds and more flower buds with age (Crane and Iwakiri, 1981).

The results of the preliminary experiments concerning establishment of mature pistachio shoot materials indicated that use of explants of glasshouse-grown plants and basal sections of summer-harvested shoots, the surface sterilization of shoots with 20% bleach solution using a shaker system and the initial incubation of cultures under dark conditions for up to three weeks could contribute positively to the

successful establishment of Pistacia shoots in vitro.

4.1.3.3 MSF from germinating P. vera and P. atlantica seeds

In Stage IB cultures, MSF was readily achieved in both P. vera (Section 4.1.2.1) and P. atlantica (Section 4.1.2.2) seeds. In the first case, 8 mg l⁻¹ BAP was optimal in stimulating shoot and bud formation. A lower concentration (1 mg l⁻¹) was, however, optimal in the latter species. The observed responses of Pistacia seeds in culture were similar to those described for other nut tree species. Levels of 1-2, 4.5 and 8 mg l⁻¹ BAP have been used to stimulate the development of axillary shoots in chestnut (Vieitez and Vieitez, 1980 in Vieitez et al., 1986), walnut (Rodriguez, 1982) and hazelnut (Rodriguez et al., 1984 in Rodriguez et al., 1989b), respectively.

Cheng et al. (1980) reported that high levels of cytokinin resulted in the elimination of apical dominance and the development of axillary buds and MSF. The fact that much higher levels of BAP were needed in the case of P. vera than in P. atlantica seeds to induce MSF might indicate a stronger apical dominance characterising seedlings of the first species as compared with the second one.

High cytokinin levels, however, increased vitrification of P. vera seedlings (grown in liquid media to overcome the problem of browning). No such symptoms were observed in P. atlantica seedlings (grown in agar-solidified media when 4 mg l⁻¹ or lower levels of BAP were used). Cytokinin availability is reported to be a determinant factor in the induction of vitrification (Pasqualetto et al., 1986; Gaspar et al., 1987) and liquid medium is confirmed to induce vitrification (Gaspar et al., 1987; Standardi and Micheli, 1988). In the case of solidified medium, cytokinin is reported to affect vitrification only at particular concentrations of the gelling agent (Debergh, 1983; Pasqualetto et al., 1986).

In addition to vitrification, STN symptoms (already mentioned in Stage IA cultures) were also observed on germinating seeds forced to produce multiple shoots. This subject is discussed in detail in Section 4.2.4.

In conclusion, optimal MSF could be achieved by initiating P. vera seedlings on liquid media supplemented with 4 mg l^{-1} BAP or even 6-8 mg l^{-1} but for much less than two weeks to limit vitrification. In the case of P. atlantica, lower levels of BAP should be used and optimal results could be obtained by initiating cultures on agar-solidified media supplemented with 0.5-1 mg l^{-1} BAP.

4.2 STAGE II : SHOOT MULTIPLICATION

In the second stage of micropropagation, initiation of multiple shoot cultures from nodal explants of P. vera (seedlings and four-year-old plant materials) and P. atlantica (seedling materials) was investigated. The effects of culture media (mineral composition), cytokinins and temperature/photoperiod on shoot multiplication were studied and investigations on shoot-tip necrosis (STN) carried out.

4.2.1 EFFECTS OF CULTURE MEDIA (MINERAL COMPOSITION)

Murashige and Skoog (1962) medium (MS) or a modification of this medium have been commonly used for shoot proliferation of Pistacia species (Barghchi and Alderson, 1983; Martinelli, 1988). Barghchi (1985a) tested MS medium against Lloyd and McCown (WPM) formulae and found no differences between the performances of shoots of P. vera when grown on either of the two media. McCown and Sellmer (1987) reported that the standard formulae commonly used for herbaceous species (e.g. MS) did not support good growth of many woody species. In view of this, a preliminary investigation was carried out to test the effects of a range of different macro- and micronutrient formulae on shoot proliferation.

Nodal segment explants of four-year-old P. vera (subcultured x 1) were grown on agar solidified media supplemented with 2 mg l⁻¹ BAP. The following media, supplemented with MS vitamins were tested:

- a) Murashige and Skoog (1962) = MS;
- b) Anderson (1978) = A;
- c) Knop (1865) = K as given by George and Sherrington (1984) supplemented with MS micronutrients;
- d) Lloyd and McCown (1981) = WPM.

Comparisons of the relative concentrations of inorganic compounds present in the above media are summarized in Table 13a.

Cultures were incubated for four weeks under standard conditions and were recultured back onto MS or A macronutrients + MS micronutrients media for a further four-week culture period.

TABLE 13a : Mineral composition of the culture media used for shoot multiplication of P. vera (mg l⁻¹) (Stage II, Section 4.2.1)

	MS (1962)	K (1865)	WPM (1981)	A (1978)	
Macronutrients					
Ca(NO3)2.4H2O	-	1,000	556	-	
NH4NO3	1,650	-	400	400	
KNO3	1,900	250	-	480	
CaCl2.2H2O	440	-	96	440	
MgSO4.7H2O	370	250	370	370	
KH2PO4	170	250	170	-	
NaH2PO4.H2O	-	-	-	380	
	MS	MS	WPM	A	MS
Micronutrients					
KI	0.83	0.83	-	0.30	0.83
H3BO3	6.20	6.20	6.20	6.20	6.20
MnSO4.4H2O	22.30	22.30	-	-	22.30
MnSO4.H2O	-	-	22.30	16.90	-
ZnSO4.7H2O	8.60	8.60	8.60	8.60	8.60
Na2MoO4.2H2O	0.25	0.25	0.25	0.25	0.25
CuSO4.5H2O	0.02(5)	0.02(5)	0.02(5)	0.02(5)	0.02(5)
CoCl2.6H2O	0.02(5)	0.02(5)	-	0.02(5)	0.02(5)
Na2.EDTA	37.30	37.30	37.30	74.50	37.30
FeSO4.7H2O	27.80	27.80	27.80	55.70	27.80

Results (Table 13b) showed the superiority of MS to all the other tested media, which did not show any real differences among them in any of the measured parameters. It was noticeable that the proportion of cultures exhibiting media browning was much lower in the case of MS medium than in any of the other media. However, callus was produced by all cultures on MS medium, whereas relatively few of the cultures grown on the other media showed callus. No callus development occurred in shoots cultured on A medium. Cultures grown on MS medium also produced shoots with faint chlorosis; this occurred in 66.7% of the cultures as compared with 38.9, 11 and 5.6% cultures for K, WPM and A media, respectively. However, shoots grown on the last three media were frequently necrotic and eventually all shoots grown on these media died.

Results of a second period of subculture for four weeks on MS or A macronutrients + MS micronutrients (Table 13c) were almost similar to those obtained in the first experiment, thus confirming the superiority of MS medium for shoot proliferation of P. vera despite the persistence of callus and faint chlorosis. It was also noticed that the low portion of shoot cultures, which showed browning when MS was used during the first subculture cycle, was reduced further such that in the second subculture browning was absent in all shoot cultures.

4.2.2 EFFECTS OF CYTOKININS

4.2.2.1 Effects of BAP alone

Different levels of BAP have been reported optimal for in vitro propagation of P. vera by several workers. A medium containing 4 mg l⁻¹ BAP was found to be optimal for seedling explants obtained from 10-day-old aseptically germinated seeds (Barghchi and Alderson, 1983). Grafted plants of one- to four-year-old plants were reported to perform better in 2 mg l⁻¹ BAP (Martinelli, 1988). An intermediate result was found when two-month-old plants were cultured in the presence of 3 mg l⁻¹ BAP (Marco-Antonio, 1984). Concerning P. atlantica, multiplication was reported to perform better in 0.7 mg l⁻¹ BAP (Martinelli, 1988).

In the following experiments, the effects of BAP on multiplication of

TABLE 13b : Effects of culture media (mineral composition) on P. vera shoot multiplication after four weeks of culture (n=18)

	Murashige			Lloyd		Anderson	Ftest	LSD	X ² test
	and Skoog	Knop	(1865) ^a	and McCown	(1981)	(1978)	(85 df)	(0.05)	(3 df)
	(1962)	(1865) ^a	(1981)						
Shoot number ^b	1.7	1.2	1.2	1.1	(P<0.001)	0.18			
Total shoot length ^c (mm)	3.6	2.5	2.4	2.4	(P<0.001)	0.20			
Total bud number ^b	3.4	1.7	1.5	1.5	(P<0.001)	0.33			
% MSF	88.9	50.0	38.9	16.7		(P<0.001)			
% Media browning	16.7	100.0	88.9	77.8		(P<0.001)			
% Callus	100.0	16.7	11.1	0.0		(P<0.001)			
% Cultures with faint Chlorosis	66.7	38.9	11.1	5.6		(P<0.001)			

^a Medium supplemented with MS micronutrients.

^b SQR transformed data.

^c Log_e "

TABLE 13c : Effects of culture media (mineral composition) on P. vera shoot multiplication after reculturing for a further four-weeks culture period (n=23)

	MS (1962)	Anderson (1978) ^a	F _{test} (44 df)	LSD (0.05)	X ² _{test} (1 df)
Numbr of shoots ^b	1.7	1.1	(P<0.001)	0.16	
Total shoot length ^c (mm)	3.7	2.3	(P<0.001)	0.16	
Total bud number ^b	3.8	1.7	(P<0.001)	0.36	
% MSF	91.3	17.4			(P<0.001)
% Media browning	0.0	95.7			(P<0.001)
% Callus	100.0	0.0			(P<0.001)
% Cultures with faint chlorosis	69.6	4.3			(P<0.001)

^a Anderson's macronutrients supplemented with MS micronutrients.

^b SQR transformed data.

^c Log_e transformed data.

explants excised from two-week-old P. vera and P. atlantica seedlings and of explants obtained from four-year-old P. vera plants (subcultured x 3) were investigated.

Nodal explants were grown on MS media either hormone-free or supplemented with BAP at 1, 2.5 or 4 mg l^{-1} in case of P. vera seedling material; 0.5, 1, 1.5 or 2 mg l^{-1} in case of P. atlantica seedling material, and 1, 2, 3, 4 or 5 mg l^{-1} in case of four-year-old P. vera material. Cultures were incubated under standard conditions for four weeks.

a) Explants of P. vera seedlings

The results presented in Table 14 showed a stimulatory effect of BAP on MSF as well as on shoot and bud numbers. All media supplemented with BAP induced MSF as compared with only 30% in hormone-free medium. BAP addition to the culture media also resulted in an improvement of the number of shoots and buds produced, the optimal responses for both parameters being obtained at 4 mg l^{-1} . Concerning shoot growth, there was some evidence to suggest that BAP may have been more stimulatory at 2.5 mg l^{-1} than at 4 mg l^{-1} . However, this difference was not significant.

b) Explants of P. atlantica seedlings

Results (Table 15) showed similar trends to those observed for P. vera with a substantial effect of BAP on total shoot length and bud number with an optimum at 1 mg l^{-1} BAP.

c) Explants of four-year-old P. vera plants

The results (Table 16) showed that BAP had a real effect on shoot growth and proliferation. MSF was induced at all BAP levels tested, and all cultures grown on media supplemented with 2 mg l^{-1} or higher developed multiple shoots. The optimum shoot number achieved was at 3-5 mg l^{-1} but this did not significantly differ from the one obtained at 2 mg l^{-1} . Concerning bud number, no significant differences in means were obtained with either 3 or 4 mg l^{-1} BAP although the latter gave the

TABLE 14 : Effects of BAP on multiplication of two-week-old P. vera seedling material after four weeks of culture (n=10)

	BAP (mg l ⁻¹)				F _{test} (36 df)	LSD (0.05)
	0.0	1.0	2.5	4.0		
Shoot number ^a	1.2	2.0	2.1	2.2	(P<0.001)	0.45
Total shoot length ^b (mm)	3.0	3.3	3.9	3.6	(P<0.05)	0.65
Total bud number ^a	2.5	3.0	3.8	4.2	(P<0.001)	0.89
% MSF	30.0	100.0	100.0	100.0		

^a SQR transformed data.

^b Log_e transformed data.

TABLE 15 : Effects of BAP on multiplication of two-week-old P. atlantica seedling material after four weeks of culture (n=6)

	BAP (mg l ⁻¹)					F _{test} (25 df)	LSD (0.05)
	0.0	1.5	1.0	1.5	2.0		
Shoot number	1.0	1.2	1.7	1.7	1.5	(P>0.05)	0.71
Total shoot length ^a (mm)	2.5	3.5	4.1	3.5	3.3	(P<0.001)	0.49
Total bud number	4.5	6.5	10.2	6.7	7.5	(P<0.01)	2.92
% MSF	0.0	16.7	50.0	50.0	66.7		

a Log_e transformed data.

TABLE 16 : Effects of BAP on multiplication of four-year-old P. vera-derived shoots after four weeks of culture (n=11)

	BAP (mg l ⁻¹)						F test (60 df)	LSD (0.05)
	0	1	2	3	4	5		
Shoot number ^a	1.0	1.1	1.9	2.0	2.0	2.0	(P<0.001)	0.28
Total shoot length ^b (mm)	3.1	3.1	3.9	3.8	3.9	3.6	(P<0.001)	0.27
Total bud number ^a	1.8	1.8	3.7	3.8	4.2	3.2	(P<0.001)	0.43
% MSF	0.0	27.3	100.0	100.0	100.0	100.0		
% Vitrification	0.0	0.0	9.1	18.2	54.5	63.6		

^a SQR transformed data.

^b Log_e transformed data.

best overall mean results. Supplementing the media with 2 to 4 mg l⁻¹ BAP gave the highest total of shoot lengths.

As with shoot proliferation, BAP affected the levels of vitrification. Symptoms were absent from media supplemented with 1 mg l⁻¹. However, at 2 mg l⁻¹ BAP, symptoms were evident and over half the cultures became vitrified on media containing 4 and 5 mg l⁻¹ BAP.

4.2.2.2 Combined effects of BAP and 2iP

In the previous experiments, the use of optimal levels of BAP for shoot multiplication resulted generally in dwarfed rosette shoots bearing small leaves. These types of shoot proved generally unsatisfactory for subsequent subculturing onto further Stage II or onto Stage III media. It has already been reported by Barghchi and Alderson (1983) that supplementation of media with either kinetin or GA₃ with or without BAP proved unbeneficial for shoot growth or proliferation of P. vera. Consequently, other alternatives for producing better growth with more healthy-looking shoots, without being detrimental to shoot proliferation, had to be investigated.

Several authors have reported the beneficial effect of 2iP (6-8-8-dimethylaminopurine), either alone or in combination with BAP, on shoot growth and proliferation of several species such as in filbert (Anderson, 1983) and apple (Landergan and Janick, 1980). In the following experiment, effects of combination of BAP and 2iP on shoot growth and proliferation of four-year-old P. vera material (subcultured x 4) were investigated.

Nodal explants were grown on MS media supplemented with 2 mg l⁻¹ BAP or 2 mg l⁻¹ 2iP, as well as on media containing the following combinations of BAP and 2iP : 1 mg l⁻¹ BAP + 1 mg l⁻¹ 2iP or 1.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ 2iP. Cultures were incubated for four weeks under standard conditions.

The results obtained (Table 17) revealed real differences between BAP and 2iP supplementation. BAP was clearly the more effective cytokinin for proliferation with more shoots, buds and total shoot length per culture being produced than on media with 2iP at the concentrations tested. However, taller shoots and more buds per shoot were obtained

TABLE 17 : Effects of combinations of BAP and 2iP on growth and proliferation of four-year-old P. vera material after four weeks of culture (n=9)

BAP (mg l ⁻¹)	2.0	1.5	1.0	0.0	F _{test}	LSD
2iP (mg l ⁻¹)	0.0	0.5	1.0	2.0	(32 df)	(0.05)
% MSF	100.0	100.0	100.0	100.0		
Total shoot number ^a	2.3	2.2	1.8	1.2	(P<0.001)	0.41
Total shoot length ^b (mm)	3.9	3.8	3.6	3.2	(P<0.05)	0.41
Mean shoot length ^b (mm)	2.2	2.3	2.4	2.5	(P>0.05)	0.33
Total bud number ^a	5.4	5.1	4.5	3.6	(P<0.001)	0.77
Average bud number per shoot	5.3	5.9	6.5	6.2	(P>0.05)	1.28

^a SQR transformed data.

^b Log_e transformed data.

with 2iP as compared with BAP; the leaves were more normal and larger in the first case, and were comparatively small in the second. When BAP was combined with 2iP in a cyto-mixture, results almost similar to those observed with BAP alone were obtained with 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} 2iP with the advantage that they had slightly taller shoots, larger leaves and higher numbers of buds per shoot. The combination in which BAP and 2iP were mixed at 1 mg l^{-1} each resulted in performances approaching those obtained with 2iP alone, except that the total bud number was significantly higher in the former case. Therefore, the general trend was that an increase in BAP and an accompanying decrease in 2iP levels produced a marked improvement in shoot proliferation and a reduction in shoot growth, and vice versa.

4.2.3 Effects of temperature/photoperiod

There are many factors other than the media constituents which may affect growth and development of plant material in culture. These factors include light and temperature (Hughes, 1981). Working with Castanea, Chauvin and Salesses (1988) concluded that environmental factors such as temperature have as much importance as chemical factors on in vitro growth. In the case of pistachio, it is only in one reference that temperature effects have been reported (Barghchi, 1985b). In that report, no significant differences in proliferation and growth of P. vera seedling material were found between cultures incubated at 20°C and those grown at 25°C . In other species such as Castanea, proliferation was, however, reported to be significantly affected by temperature (Chauvin and Salesses, 1988). In the following experiment, the effects of temperature/photoperiod on shoot growth and proliferation of four-year-old derived Stage II P. vera cultures (subcultured x 4) were investigated.

Nodal segments were grown on MS medium supplemented with 3 mg l^{-1} BAP. Cultures were incubated for four weeks under the following available environmental conditions : $19^{\circ}\text{C}/16\text{h}$, $25^{\circ}\text{C}/16\text{h}$ and $29^{\circ}\text{C}/12\text{h}$ day temperature/photoperiod combinations.

The results presented in Table (18) showed a very strong evidence of real effects of temperature/photoperiod on growth and proliferation of

TABLE 18 : Effects of temperature/photoperiod on growth and proliferation of four-year-old P. vera after four weeks of culture (n=10)

	Temperature/photoperiod			F _{test}	LSD
	19°C/16h	25°C/16h	29°C/12h	(27 df)	(0.05)
Shoot number	3.1	8.6	10.0	(P<0.001)	1.78
Total shoot length (mm)	23.9	83.8	85.9	(P<0.001)	15.39
Mean shoot length (mm)	8.2	9.9	8.7	(P>0.05)	2.00
Total bud number	21.6	65.2	66.4	(P<0.001)	10.38
Fresh weight ^a (g)	6.3	7.2	7.8	(P<0.001)	0.41
% MSF	80.0	100.0	100.0		

^a Log_e transformed data (Fresh weight per culture)

four-year-old P. vera material. The best results were obtained at 29°C/12h but these were not significantly different from those observed at 25°C/16h. The lowest shoot proliferation and growth were obtained at 19°C/16h, the performances of cultures being significantly different from those obtained at 25°C/16h and 29°C/12h. Noticeably, there was no real difference in mean shoot length of shoot cultures grown at the different combinations of environmental conditions.

The results obtained suggested that incubation of cultures at 29°C/12h and 25°C/16h provided suitable conditions for good growth and proliferation of pistachio shoots in vitro.

4.2.4 INVESTIGATIONS OF SHOOT-TIP NECROSIS (STN)

Apart from vitrification, STN was the major problem observed, during the course of the present work, in growing Pistacia cultures under in vitro conditions. This physiological disorder was persistent through the first three stages of culture; it was observed on seedlings of both P. vera and P. atlantica germinated in vitro (Stage IA) or when seedlings were forced to induce multiple shoots (Stage IB), on actively growing shoot cultures (Stage II) and on in vitro rooted shoots (Stage III). No symptoms developed on glasshouse-grown seedlings. Several authors also reported such a problem in in vitro cultures in other species such as potato (Sha et al., 1985), almond (Rugini et al., 1986) and chestnut (Vieitez et al., 1989). In pistachio shoot cultures, similar symptoms have been reported on P. vera (Barghchi and Alderson, 1985), P. terebinthus (Pontikis, 1985) and on P. integerrima (Martinelli, 1988). Even in the case of field- or glasshouse-grown plants of several different species it has been reported that plants can exhibit similar symptoms referred to variously as STN, tipburn or shoot dieback; strawberry (Mason and Gutteridge, 1974), lettuce (Collier and Tibbitts, 1984) and apple (Kotze, 1979) are examples of such species.

As to the cause(s) of this physiological disorder, several hypotheses have been proposed to explain the development of STN, but deficiencies of boron (Hoagland, 1933 in Mason and Guttridge, 1974) and particularly of calcium (Sha et al., 1985; McCown and Sellmer, 1987) have been

reported to be the most likely factors involved.

In a recent review of pistachio micropropagation, Barghchi and Alderson (1989) suggested that deficiencies of boron or calcium are most likely to be the causes of STN. Martinelli (person. comm., 1989) was of the opinion that it is physical rather than chemical factors that are most likely to be responsible for STN in in vitro grown material of pistachio. However, no detailed experimental information has been presented in either of these cases to prove unequivocally that STN is caused by calcium/boron or by physical characteristics of in vitro culture systems. Further investigations were needed, therefore, to obtain more information on the probable causes of STN especially since a satisfactory solution to this problem has been emphasized by Martinelli (person. comm., 1989) as being crucial before successful commercial micropropagation blueprints of Pistacia species can be attained.

The work on STN carried out in the current study is reported in four parts: firstly, the sequence of visible events leading to the development of STN symptoms in Stage I pistachio cultures were investigated; secondly, a series of investigations on the effects of a range of cultural, environmental, physiological and genotypic factors on STN incidence were carried out; thirdly, a characterization of STN symptom development at the tissue and cellular levels using light and TEM microscopy and fourthly, having established the nature of STN problem, attempts were then made to develop cultural strategies which could reduce or eliminate the effects of this problem in pistachio micropropagation.

4.2.4.1 Visual description and sequential development of STN symptoms

Seedlings of P. vera grown in vitro on half strength MS liquid medium developed STN in the following typical sequence :

Phase 1 - Day 12. Early visible symptoms consisting of pale brown necrosis at leaf tips developed at the edges of the youngest leaves which curled downwards and inwards.

Phase 2 = Day 15. Approximately three days after appearance of the above symptoms meristem-tips darken and die back (Plate 2).

Phase 3 = Day 16 and onwards. Darkened necrotic areas progressively enlarge and spread downwards along the stem and axillary branches begin to develop as a result of death of apical tip (Plate 3).

Phase 4 = Complete death of shoots by 28 days.

A similar but slower rate by about 3 to 5 days of development of STN symptoms was observed in P. atlantica materials.

4.2.4.2 Effects of cultural, environmental and other factors

In the previous experiments the incidence of STN development was found to be affected by several cultural and environmental factors. In the present section, a summary of the observed effects of the factors studied on STN is presented:

a) Effects of mineral medium strength

P. atlantica seedlings developing on agar-solidified media (Stage IA, Section 4.1.1.1d) showed differences in STN symptoms following growth on different strengths of media (mineral composition). STN levels were reduced from 18.2 to 8.3% by growing seedlings on $1/10$ x MS medium instead of DDW (control), but was increased up to 45.5 and 50.0% after 20 days of culture when $1/4$ and $1/2$ x MS media were used, respectively (Figure 4).

b) Effects of cytokinin type (BAP and/or 2iP)

The natural cytokinin 2iP, when used alone in the culture medium (Stage II, Section 4.2.2.2), resulted in the lowest level of STN (11.1%), and this increased with relative increase in BAP and decrease

PLATE 2 : STN symptoms showing necrosis of the youngest leaves and die back of the meristem-tip on actively growing shoots derived from four-year-old P. vera (Phase 2).

PLATE 3 : STN symptoms showing necrotic and dark areas progressively expanding downwards on the stem (Right) and the development of axillary branching (Left) on P. atlantica seedlings (Phase 3).



in 2iP levels used in the media; 22.2% was obtained using 1 mg l^{-1} BAP + 1 mg l^{-1} 2iP as compared with 33.3% when either a 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} 2iP combination or BAP alone at 2 mg l^{-1} were used (Figure 5).

c) Effects of BAP levels

Supplementation of 2 mg l^{-1} BAP to hormone-free medium reduced the occurrence of STN on P. vera seedlings from 66.7% to 14.3% in the experiment aimed to induce MSF (Stage IB, Section 4.1.2.1). However, symptoms again increased up to 55.6% at 4 mg l^{-1} BAP, concentrations above which cultures showed STN at levels of only 30.7 - 40% (Figure 6). Similarly, in the case of four-year-old P. vera shoots, the development of STN followed a trend as in the seedlings with an optimum STN level of 72.7% being obtained on media with 3 mg l^{-1} BAP after four weeks of culture (Stage II, Section 4.2.2.1c) (Figure 7).

d) Effects of solidifying agent

Explants of P. atlantica seedlings and four-year-old P. vera materials were cultured on proliferation media containing 1 mg l^{-1} BAP (Stage II). Media were solidified either with agar (7 g l^{-1}) or with phytogel (3.5 g l^{-1}) and cultures were incubated under standard conditions.

Results (Figure 8) showed that the use of phytogel resulted in a significant increase in STN, especially in the case of P. vera. STN increased from 27.3 to 83.3% and from 31.6 to 67.9% in the case of P. vera and P. atlantica, respectively. Noticeably, the results obtained (Table 19) provided a very strong evidence of a real improvement of shoot growth and development following culture on phytogel at 3.5 g l^{-1} as compared with agar at 7 g l^{-1} .

e) Effects of different temperature/photoperiod regimes

The frequencies of STN development in four-year-old P. vera shoots incubated at $19^{\circ}\text{C}/16\text{h}$, $25^{\circ}\text{C}/16\text{h}$ and $29^{\circ}\text{C}/12\text{h}$ photoperiods (Stage II,

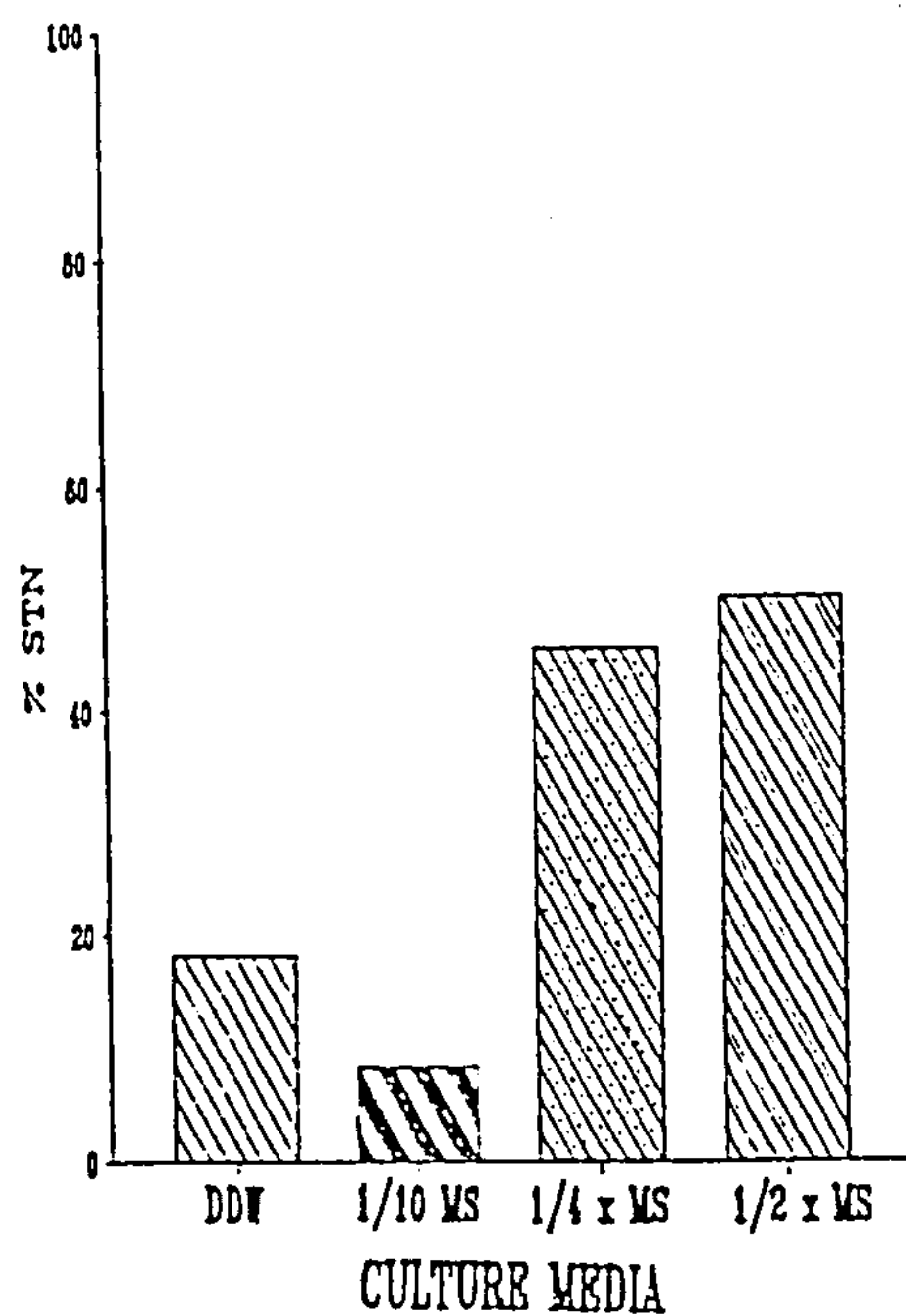


FIGURE 4 : Effects of mineral medium strength on STN in *P. atlantica* seedlings after 20 days of culture on agar solidified media (Stage IA) (n=18-20).

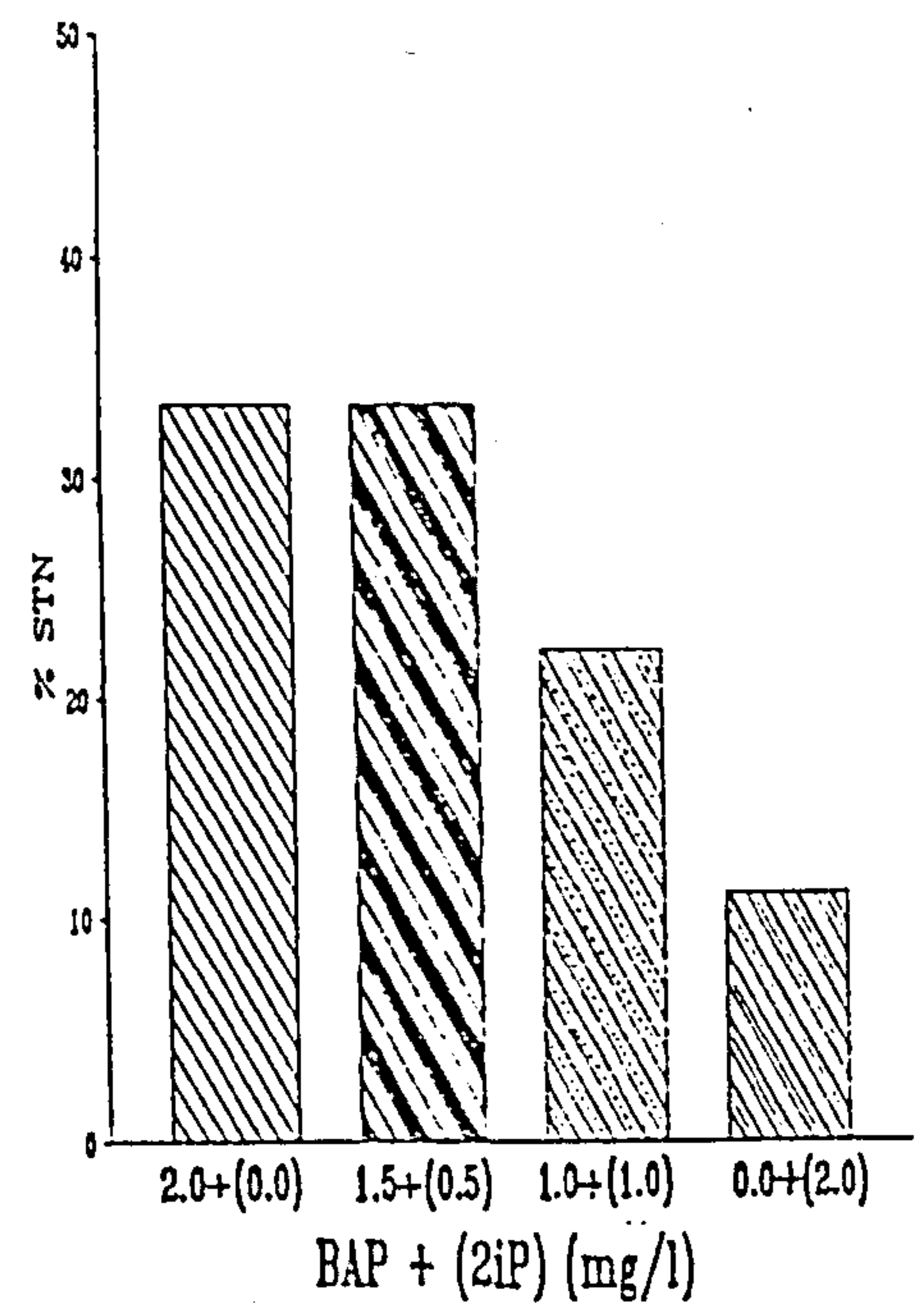


FIGURE 5 : Effects of cytokinin type (BAP and/or 2iP) on STN development in shoot cultures of four-year-old *P. vera* after 28 days in culture (Stage II) (n=9).

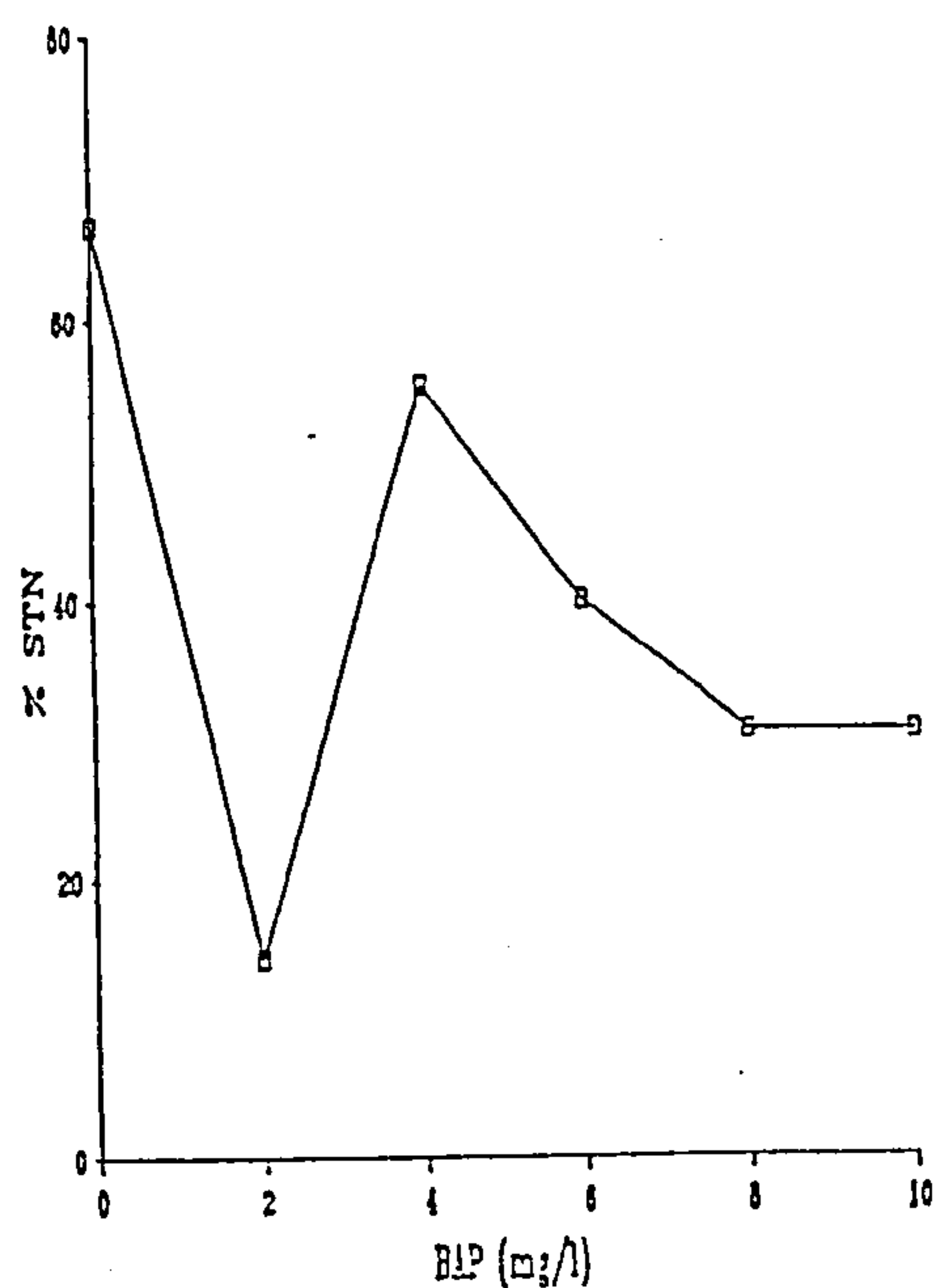


FIGURE 6 : Effects of BAP levels on STN in *P. vera* germinating seeds after 14 days of culture (Stage Ia) (n=10).

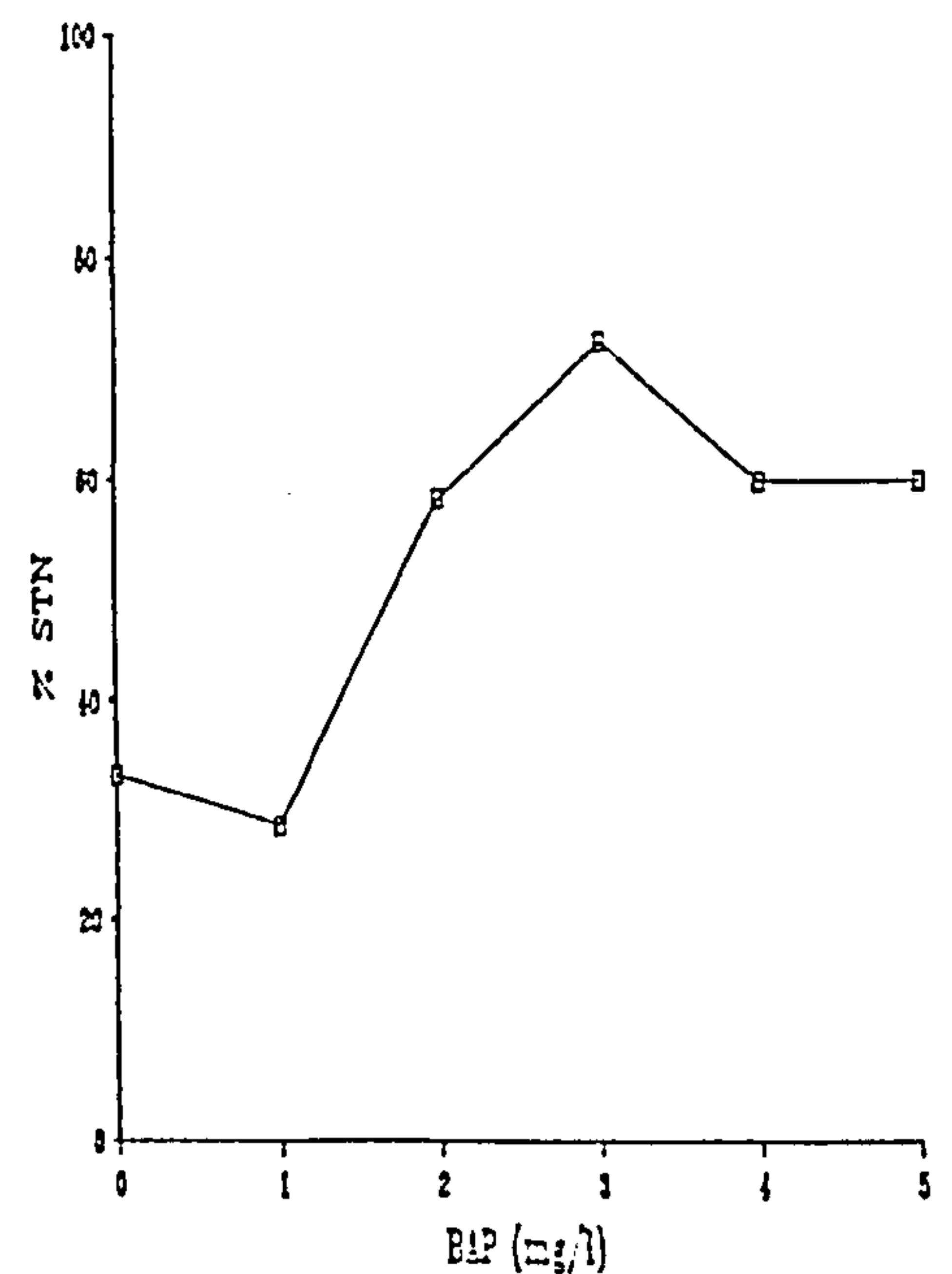


FIGURE 7 : Effects of BAP levels on STN in four-year-old *P. vera* derived microshoots after 28 days of culture (Stage II) (n=11).

TABLE 19 : Effects of agar (7. gl⁻¹) vs phytozel (35gl⁻¹) on shoot growth and proliferation of P. atlantica seedling shoots after four weeks of culture (n=28)

	Phytozel	Agar	F _{test} (54 df)	LSD (0.05)
Shoot number ^a	1.7	1.1	(P<0.001)	0.18
Total shoot length ^b (mm)	4.0	2.6	(P<0.001)	0.29
Total bud number ^a	4.2	2.5	(P<0.001)	0.36

^a SQR transformed data.
^b Log_e transformed data.

Section 4.2.3) suggested that STN was clearly affected by the environmental conditions under which cultures were incubated (Figure 9). No STN symptoms developed at 19°C/16h, whereas, 50% of the cultures incubated at 25°C/16h showed STN. In the case of cultures grown at 29°C/12h, STN was observed in only 20% of the cultures. Noticeably, STN symptoms continued to develop in time, thus increasing from 6.3 to 37.5% and then to 50.0% by 9, 21 and 28 days of incubation at 25°C/16h. By contrast, when cultures were grown at 29°C/12h the development of STN seemed to stabilize after 21 days at 20%.

f) Effects of vessel closure type

Four-year-old P. vera shoots were grown on MS medium supplemented with 1 mg l⁻¹ 2iP at a culture density of three shoots per vessel. Jars (100 ml) covered with 'Magenta' plastic lids, either unsealed or sealed with nescofilm, were incubated at 25°C/16h for four weeks.

Results (Figure 10) showed that the closure type of vessels clearly stimulated the development of STN. No symptoms developed on shoots cultured in unsealed cultures by three weeks of incubation; only 16.7% shoots developed STN one week later as compared with 54.5% in the case of shoots cultured in sealed vessels.

g) Effects of presence or absence of roots

Shoot-tips of explants were excised from P. vera seedlings grown in vitro and were induced to root on 1/2 x MS medium supplemented with 2.5 mg l⁻¹ IBA. At first, cultures were incubated for one week in darkness before they were transferred into light (16h). Once the root-tips were visible, shoots were subcultured on hormone-free medium.

Data collected (Figure 11) showed that both rooted and non-rooted shoots developed STN. However, only 25% of rooted shoots showed symptoms of STN while in the other group 64.7% showed STN symptoms at 11 days after incubation. By 14 days, these levels had increased to 60 and 100%, respectively.

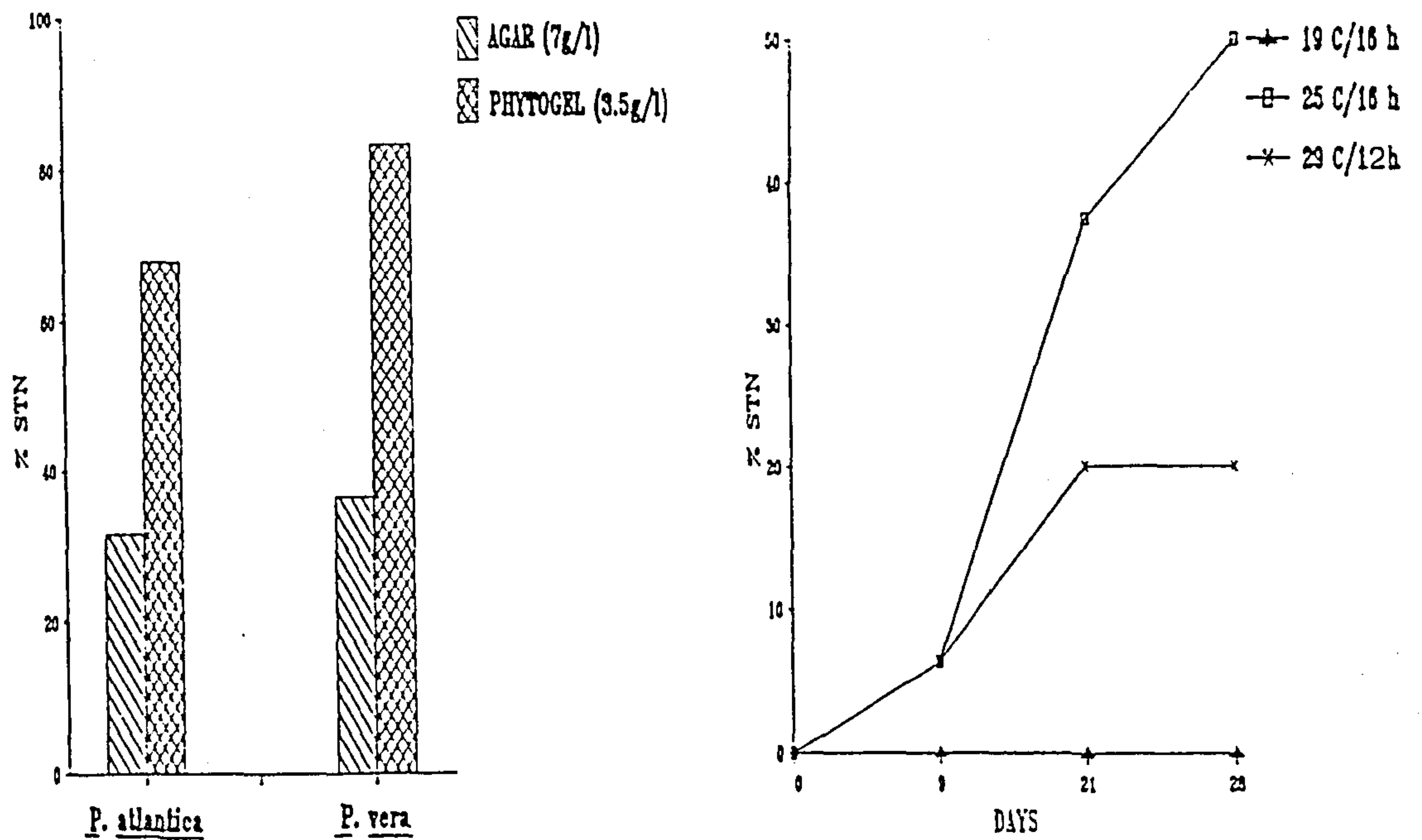


FIGURE 8 : Effects of solidifying agent (agar vs phytoGel) on STN in *P. atlantica* seedling and four-year-old *P. vera* derived microshoots after 28 days of culture (Stage II) (n=28). χ^2 test (1 df) : (P<0.05).

FIGURE 9 : Effects of environmental conditions (temperature/ photoperiod) on STN in four-year-old *P. vera* derived microshoots after 9, 21 and 28 days of culture (Stage II) (n=10).

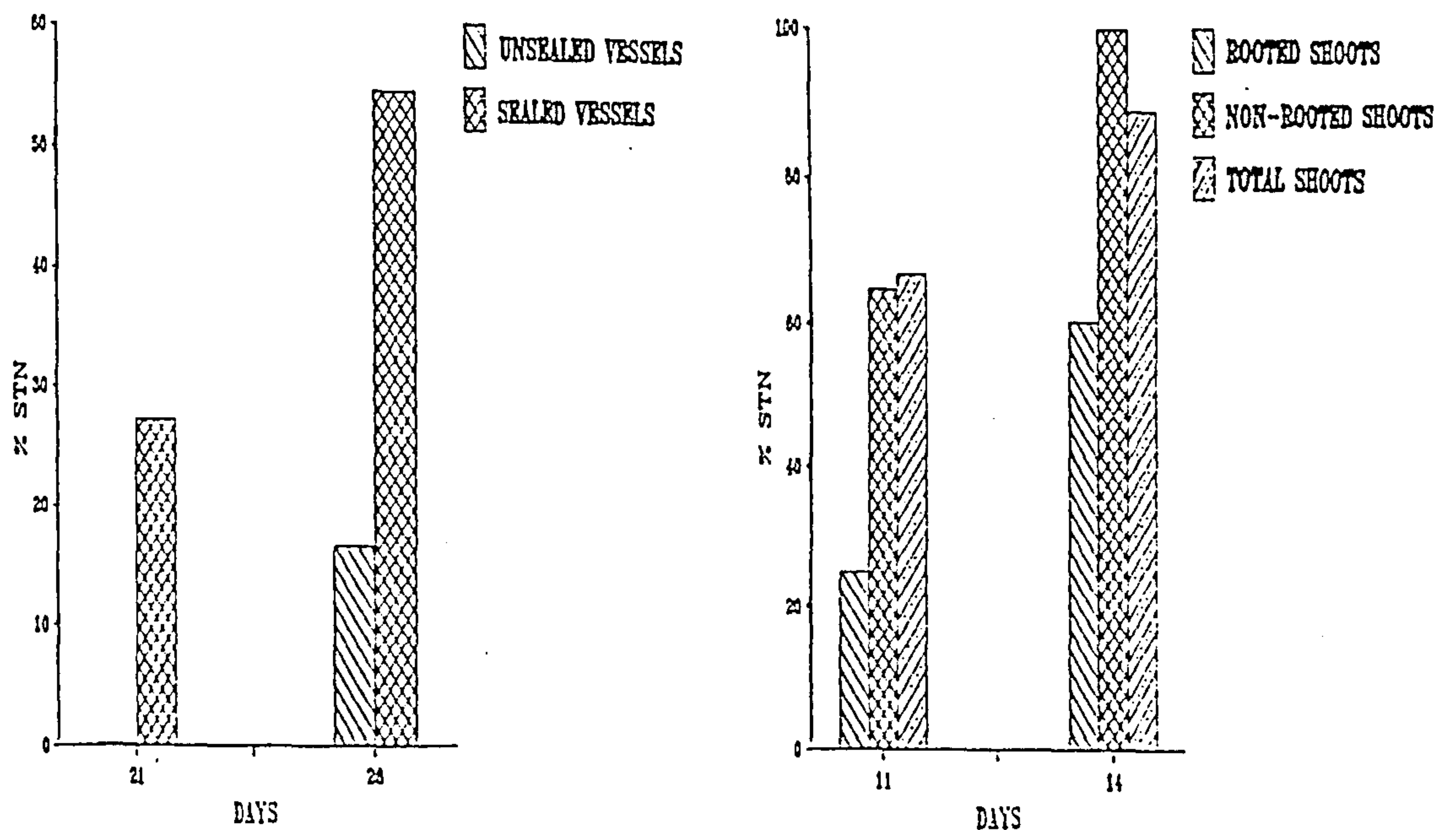


FIGURE 10 : Effects of vessel closure on STN in four-year-old *P. vera* derived microshoots after 21 and 28 days of culture (Stage II) (n=11-12). χ^2 test (1 df) : (P>0.05).

FIGURE 11 : Incidences of STN in rooted and non-rooted shoot-tip explants of *P. vera* seedlings after 11 and 14 days of culture (Stage III) (n=18).

h) Genotype of cultured material

Explants excised from P. vera and P. atlantica seedlings raised in vitro and then grown on a shoot multiplication medium containing respectively, 3 and 1 mg l⁻¹ BAP (Stage II, Section 4.2.2.1.2a,b) expressed different sensitivities to STN; the highest level of symptoms being observed on the former species. After four weeks of incubation under standard conditions, 61.5% P. vera cultures showed STN symptoms as compared with only 36.4% in the case of P. atlantica.

i) Effects of mother-stock age (seedlings vs four-year-old shoots)

Cultures of four-year-old P. vera shoots grown on proliferation media (Stage II, Section 4.2.2.1a,c) expressed higher levels of STN than those of P. vera seedling material in which levels of 64.7 and 38.9% were observed, respectively.

The above results show clearly that STN symptoms first appear on the youngest leaves, then on the meristem-tip before they progress down the stem and death of the shoot and development of axillary branches result. Axillary shoots also develop STN and branch again in a similar and progressive manner. This condition was affected by several cultural, environmental, genotypic and physiological factors; some conditions induced STN in a more rapid manner and conditions which supported optimal growth appeared the most conducive to the development of STN symptoms.

4.2.4.3 Histological studies

a) Light microscopy (LM)

Apices as well as young leaf tissues of P. vera and P. atlantica seedlings grown in vitro under standard conditions and which showed typical STN symptoms were excised and prepared for LM as previously described in Section (3.7.2). Shoot materials at Phase 2 of STN development were mainly used for this investigation. A batch of unaffected shoots from the same cultures were also treated in the same way as controls. Longitudinal as well as transverse sections of the

above organs and tissues when examined under a light microscope showed that early signs of STN consisted of disorganization of individual cells in the cortex as well as in the pith of shoot-tips, and in both palisade and spongy parenchyma in the case of leaves. Consequently, areas with disorganized cells were selected and prepared for the ultrastructural studies to investigate STN at the cellular level.

b) Transmission electron microscopy (TEM)

Attempts to fix dehydrate and embed pistachio tissues were successful in preserving the structuring of Pistacia tissues, thus providing suitable specimens for examination under a TEM. Ultrathin sections of mesophyll cells showed that while the middle lamella was clearly discernible, the cytoplasm of these cells was electron dense. This problem was encountered with the P. vera material in particular, and this made it difficult to identify inclusions and organelles in materials of this species. Consequently, anti-browning compounds (1% caffeine or 0.5% ascorbic acid) were added to the first fixative solution used for specimen preparation. Both of these supplements prevented the excessive staining of P. vera material and allowed a better investigation to be carried out on the ultrastructural features of P. vera cells. The TEM study showed that the features observed were similar, regardless of the type of tissues (cortex, pith and parenchyma) and of species (P. vera vs P. atlantica). The main abnormal structural feature observed in cortex and pith (of shoot-tips) or mesophyll parenchyma (of leaves) showing early signs of STN consisted of invaginations of the plasmalemma. These were not present in ultra-thin sections of control materials (Plate 4). In STN-affected cells, the plasmalemma was displaced away from its normal position against the cell wall. Small granulated bodies also accumulated within the invaginations formed (Plate 5). A further stage of disintegration consisted of the accumulation of degenerative vesicles and the disintegration of tonoplasts and plasmalemma (Plate 6). Following this apparent breakdown of compartmentation, nuclei and chloroplasts displayed abnormal shapes and they appeared to be disintegrating. More degenerative vesicles were produced and dense inclusions appeared as STN progressed; these, together with other cytoplasm components and fragments of membranes and damaged organelles,

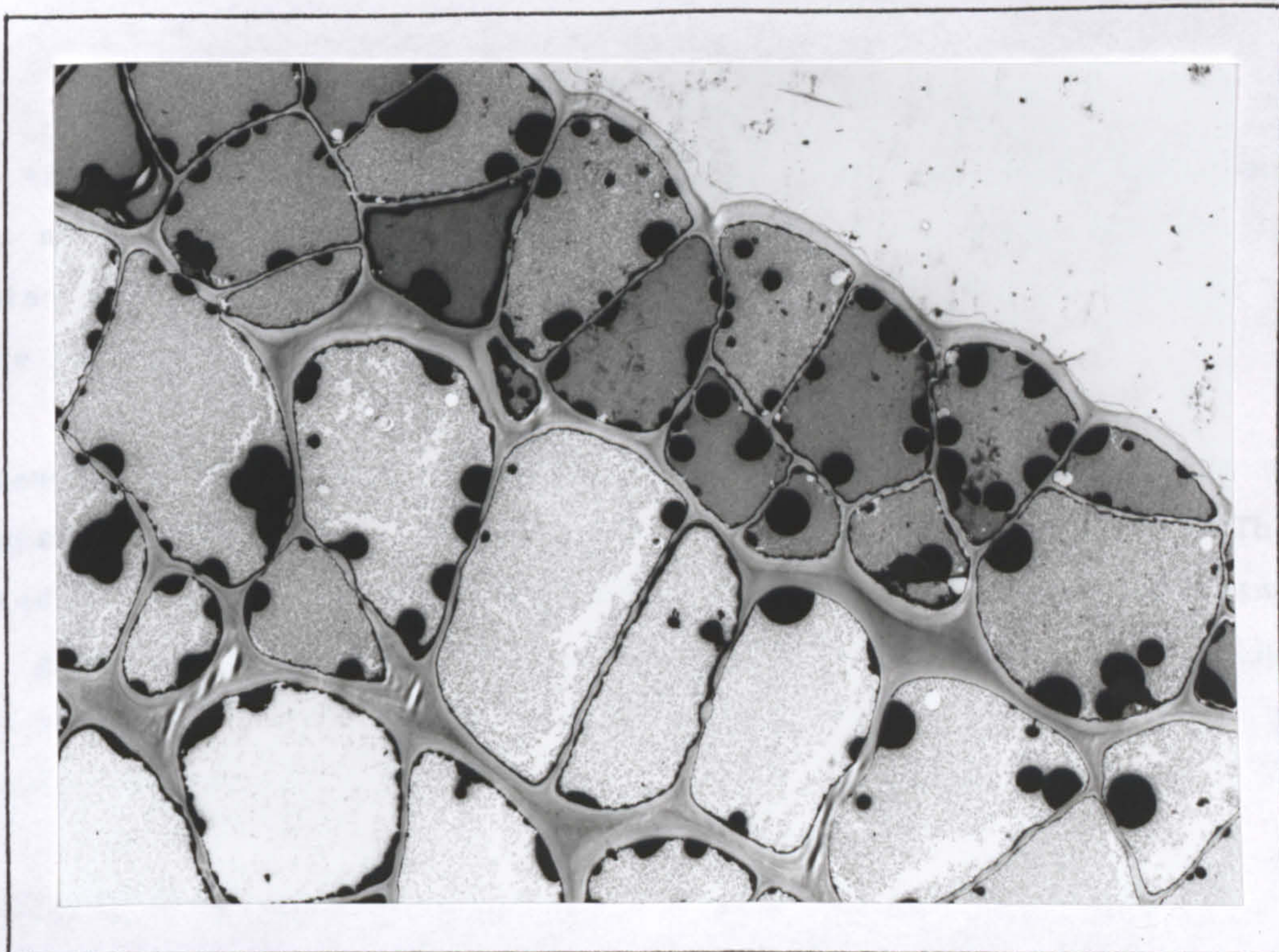


PLATE 4 : Apparently healthy cells with plasmalemma in its normal position against the cell wall (LG section, cortex of P. vera shoot-tip, TEM, X 1,500).

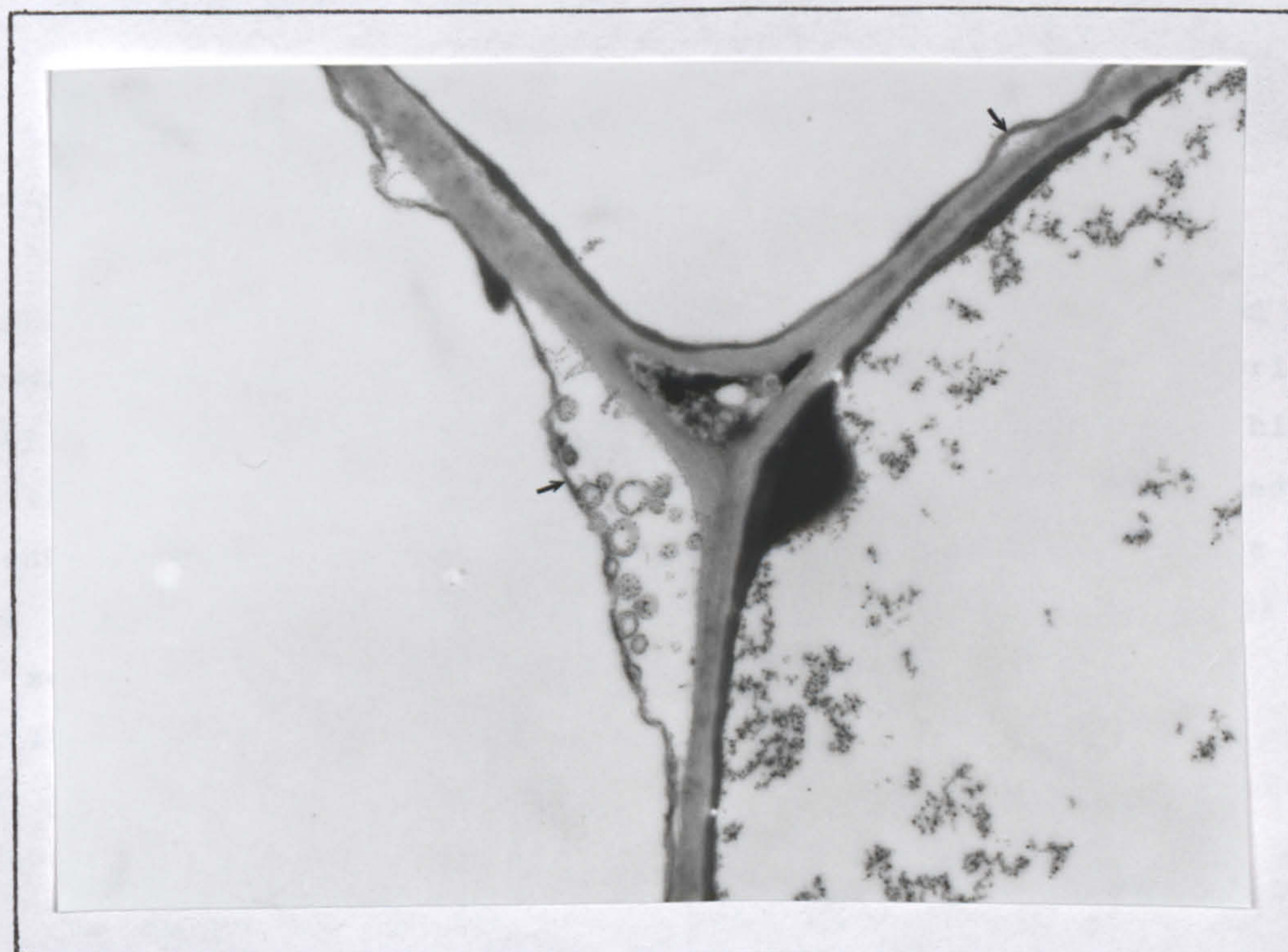


PLATE 5 : Apparently healthy cell with plasmalemma against the cell wall (Right) neighbouring cells showing initial symptoms of invaginations of the plasmalemma and pulling away from its normal position (Arrows). Accumulation of small bodies in the formed invaginations (LG section, cortex of P. vera short-tip, TEM, X 18,000).

filled the intra-cellular space of cells. In the course of these events, middle lamellae developed darker staining areas (Plate 7). Gaps also developed in the cell wall along and/or at the points of juncture between cells (Plate 6).

The present TEM studies revealed that extensive disintegration of membranes and organelles occurred in cells affected by STN. This consisted of breaking up of plasmalemma, tonoplast and other membranes and a general disorganization of chloroplasts, nuclei and other structures. Cell walls appeared also to degenerate with gaps appearing along their lengths as tissue structure degenerated.

4.2.4.4 Effects of calcium supplementation to the culture medium

The results of previously reported work on STN, the ultrastructural studies and the observations made on shoot cultures (Sections 4.2.4.2,3), suggested that calcium (Ca) deficiency in shoot-tips and rapidly growing leaf-tips could have been involved in the development of STN. Consequently, the following experiment was conducted in order to investigate the effects of Ca on STN through supplementing this ion at various levels in the culture medium.

Since calcium chloride has been reported to result in leaf yellowing of the leaves, weak stems and even death of tissues because of chloride toxicity (McCown and Sellmer, 1987), and since supplementing high levels of calcium has not been possible without modifying other media components (Sha et al., 1985) the use of calcium gluconate (which is an organic source of Ca), suggested by Zeldin and McCown (1986) as a useful source of Ca for plant tissue culture media, was adopted. In the present experiment, Stage II cultures derived from four-year- old P. vera shoots (subcultured x 5) were cultured on MS medium which was modified so as to give four levels of Ca which were 0.3, 3, 15 and 30 mM. In the former two cases, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was used in media and in the last two (i.e., in those cases where supplementation of Ca was above the usual MS level of 3 mM) raised Ca levels were provided by use of calcium gluconate (Sigma). Culture media were supplemented with 3 mg l^{-1} BAP and cultures were grown for five weeks under standard conditions.

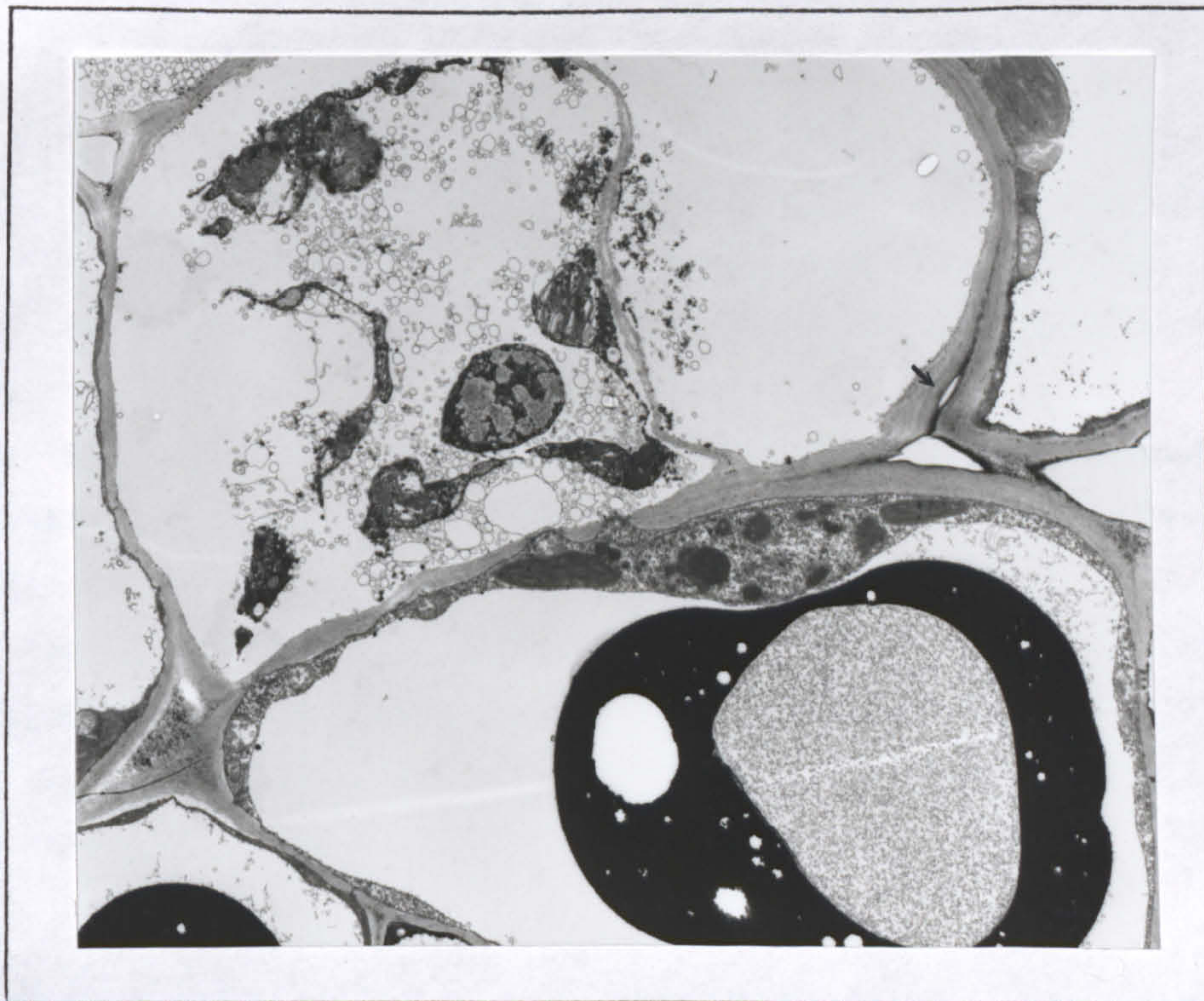


PLATE 6 : Disintegrating cell (Top) adjacent to a healthy cell (Bottom), showing breakdown of compartmentation and disintegration of tonoplast and plasmalemma membranes. Degenerative vesicles accumulating and nucleus and chloroplasts disintegrating. The middle lamella contains gaps as well as a darkened area (Arrow). (LG section, cortex of P. vera shoot-tip, TEM, X 45,000).

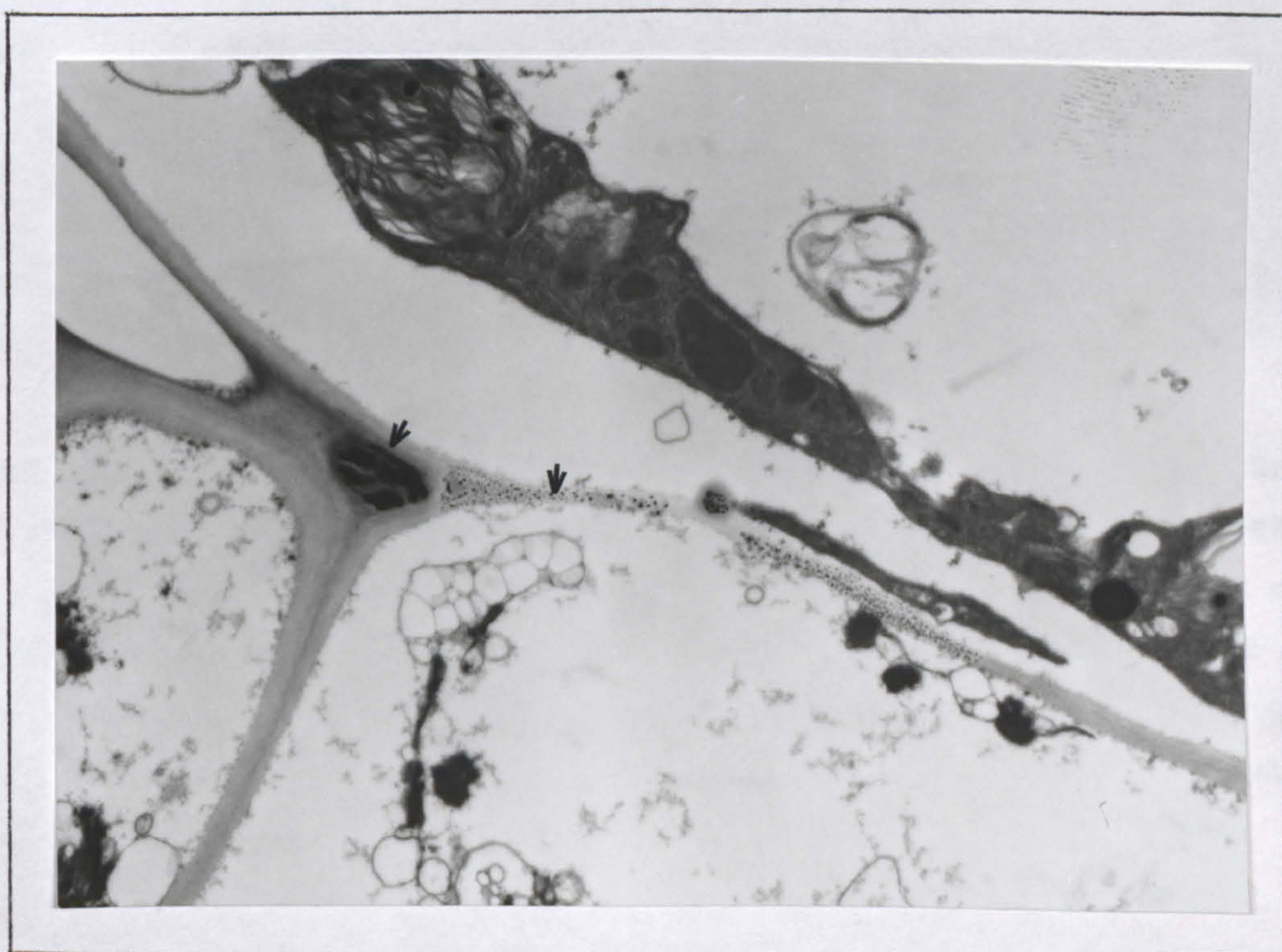


PLATE 7 : Disintegrating middle lamella. Regions in the wall and in the junction between cells appear opaque (LG section, cortex of P. vera shoot-tip, TEM, X 10,500).

Although calcium generally improved the shoot numbers, the total shoot lengths and the bud numbers present in the cultures, no evidence was obtained for statistically significant differences between these parameters in cultures grown on the four tested levels of Ca (Table 20).

Concerning STN, the results presented in Figure 12, showed that supplementation of media with Ca as high as 30 mM did not prevent the development of STN. As many as 50% cultures grown on 0.3 mM Ca developed the symptoms as early as five days after culture. When the four treatments were compared with each other, however, a significant reduction in STN was obtained when levels of Ca were raised in the media (Test for linear trends: $P < 0.05$).

4.2.4.5 Vapour pressure deficit (VPD) measurements

- Since calcium-related disorders have been reported to occur even in the case of sufficient supplies of Ca (Shear, 1975), and because of the particular dependence of Ca movement in plants on transpiration (Stebbins and Dewey, 1972; Bangerth, 1979), the hypothesis that high humidity levels within the culture vessels might reduce the transpiration rates of tissues and hence Ca uptake or transport seemed most probable. In order to test this hypothesis, VPD was measured and stomata opening was examined (using SEM) under the three different environmental conditions, i.e., 19°C/16h, 25°C/16h and 29°C/12h, known to affect the incidence of STN in pistacho shoot cultures (Section 4.2.4.2e).

Relative humidity within tubes was measured by placing a capacitance sensor inside a culture vessel containing a shoot, and readings taken using a hygrometer (Hygrotest Testo 6400, Testotem Ltd) after 30 min stabilization of humidity levels within the vessel. Temperatures of the air space and of the air at the leaf surface level within the tube were measured using a thermocouple (Copper constantan). Meter readings were used to calculate vapour pressure deficit (VPD) as follows :

TABLE 20 : Effects of Calcium level in modified MS medium on growth and multiplication of four-year-old P.vera-derived shoots after five weeks of culture (n=16)

	Calcium (mM)				Ftest (32 df)	LSD (0.05)
	0.3	3	15	30		
% MSF	100.0	100.0	100.0	100.0		
Shoot number ^a	1.8	2.0	2.1	2.2	(P>0.05)	0.48
Total shoot length ^b (mm)	3.4	3.7	3.9	4.0	(P>0.05)	0.50
Total bud number	5.4	6.2	6.3	6.8	(P>0.05)	1.26

^a SQR transformed data

^b Log_e transformed data

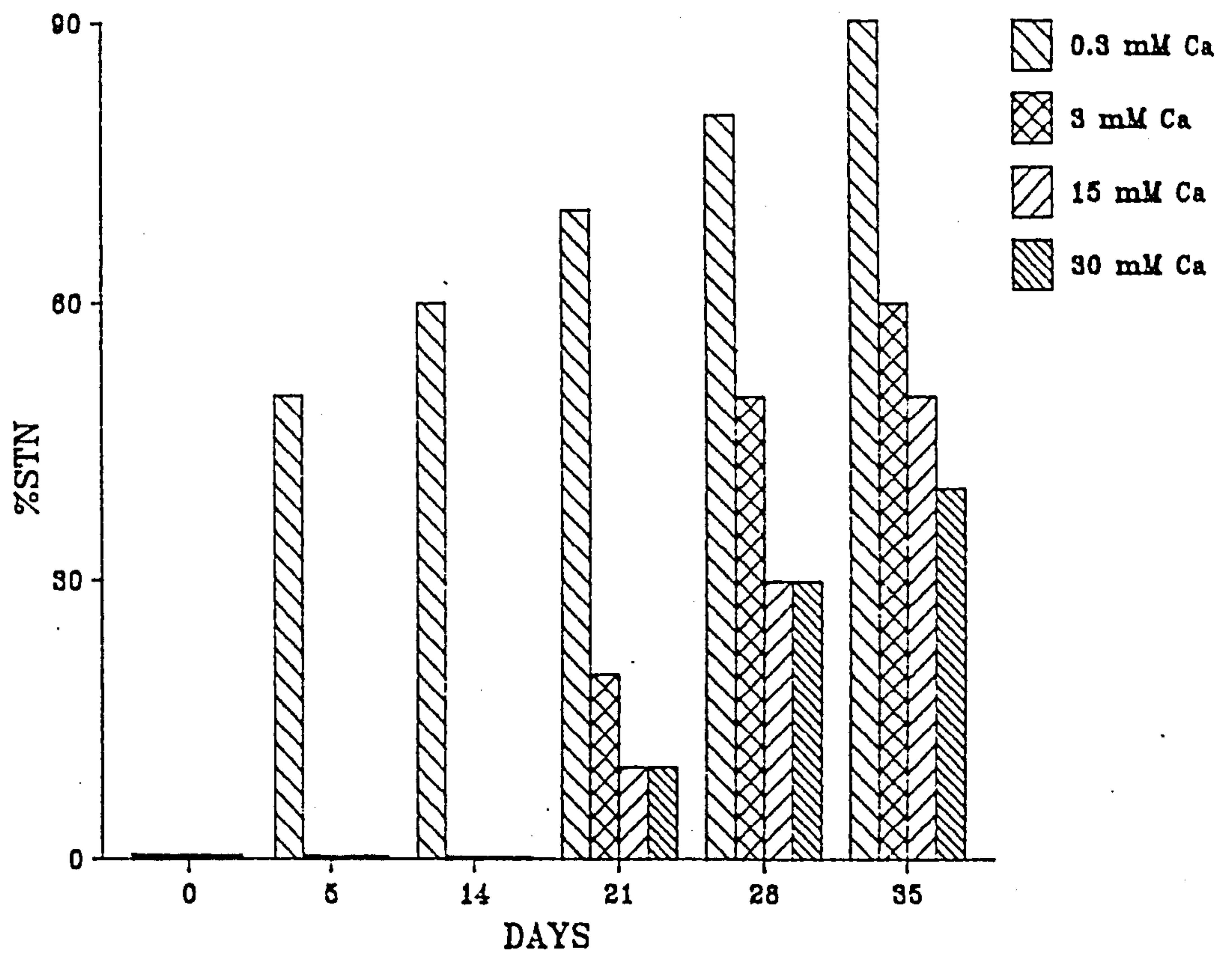


FIGURE 12 : Development of STN in four-year-old *P. vera* derived microshoots incubated at 25°C/16h on media containing increasing levels of Ca (n=16).

VPD = Saturated vapour pressure (SVP) - actual vapour pressure (AVP)

where SVP (air temperature) was obtained from meteorological tables (Robert, 1966) and AVP = SVP (tissue temperature) x measured relative humidity.

Results (Table 21) showed that VPD was low in cultures incubated at 25°C/16h (0.6 mb) and high at 29°C/12h (2.3 mb). Intermediate values were obtained at 19°C/16h (1.6 mb).

Concerning stomata characteristics, more circular and widely open stomata were observed on leaves of shoot cultures held at 25°C/16h (Plate 8), while those of cultures grown at 29°C/12h (Plate 9) and 19°C/16h (Plate 10) showed elliptical and apparently regular stomata. Measurements of representative lengths and widths of stomatal pores of four-year-old P. vera-derived shoots grown under the three environmental conditions are shown in Table 21.

This data showed that in those conditions where STN tended to be highest, VPD values were low and stomatal apertures were widest.

4.2.4.6 Effects of "feeding" shoot-tips and emerging leaves with Ca

Foliar application of Ca has been practiced by several workers to control Ca-related disorders in plants (Sharples and Kirby, 1971; Dixon et al., 1973). Since applications of sprays in vitro might tend to result in infections of aseptic cultures, a technique was adopted whereby liquid medium containing raised levels of Ca was applied to the shoot-tips and emerging leaves by immersing them in the medium through periodic inversion of tubes to allow a better supply of liquid media to the top parts of shoots.

P. vera seedlings were grown under standard conditions on $1/2$ x MS liquid medium supplemented with 15 mM of calcium gluconate. After nine days of incubation, culture tubes were either maintained in the usual upright position or were inverted three times to ensure that shoot-tips and leaves of cultures were moistened with the solution. This practice was repeated regularly thereafter every three days. STN levels in both batches of cultures were then assessed at 10, 16, 20 and 28 days.

TABLE 21 : Effects of environmental conditions on VPD and stomata characteristics of four-year-old P. vera shoots after four weeks of culture (n=3)

	Environmental conditions		
	19°C/16h	25°C/16h	29°C/12h
Vapour pressure deficit (mb)	1.6 ± 0.01	0.6 ± 0.04	2.3 ± 0.02
Stomata length (internal) (µm)	13.7 ± 0.65	14.4 ± 0.57	12.1 ± 0.20
Stomata width (internal) (µm)	3.2 ± 0.40	8.7 ± 0.78	5.4 ± 0.68

Mean ± SE

PLATE 8-10 : Scanning electron micrographs of an abaxial leaf surface of four-year-old P. vera-derived shoots grown in vitro showing stomata pores. (8) : large, more circular and widely open stomata of cultures incubated at 25°C/16h. (9) and 10) : elliptical and apparently regular stomata of cultures incubated at 29°C/12h and 19°C/16h, respectively, (X 2,500).

PLATE 8

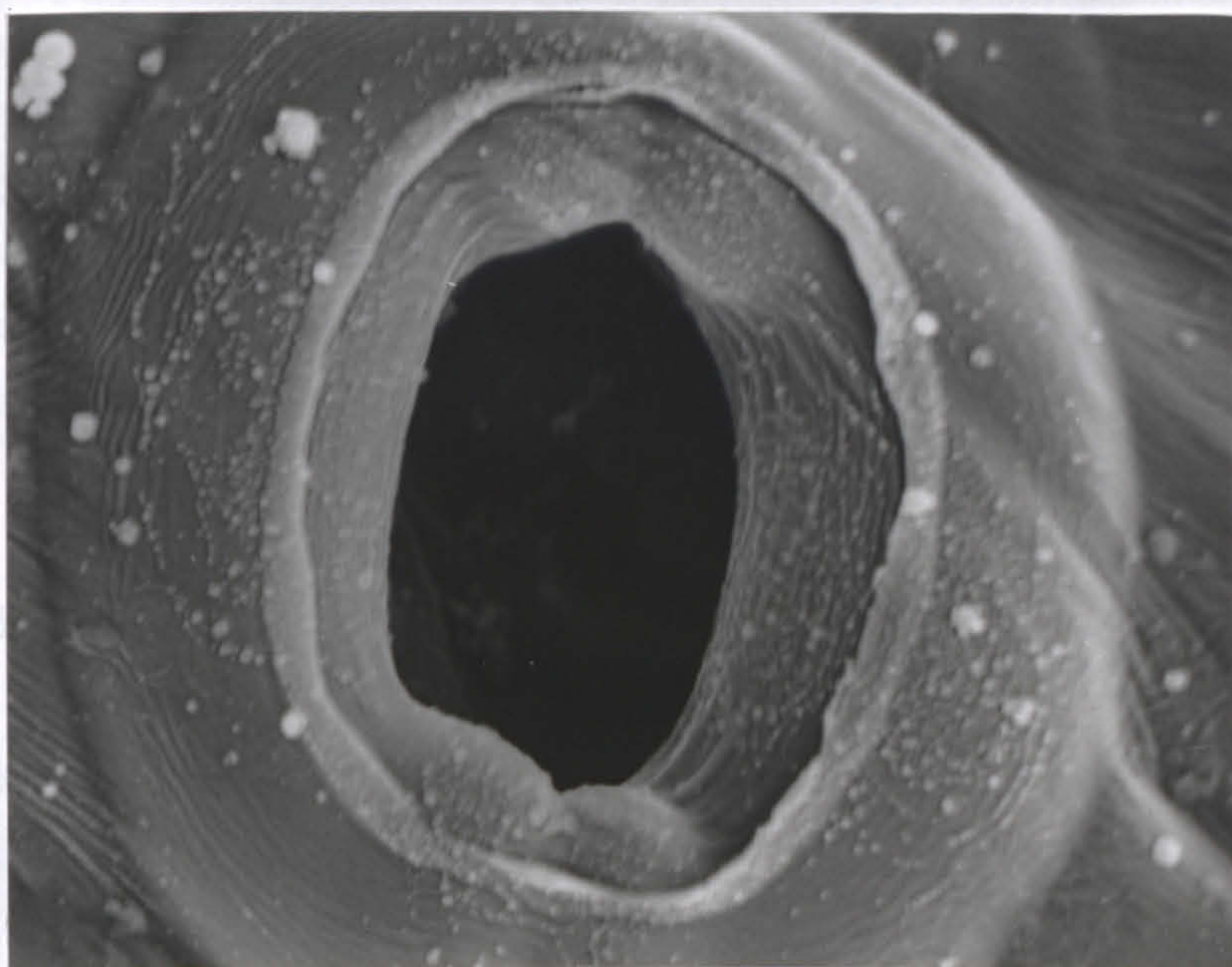


PLATE 9

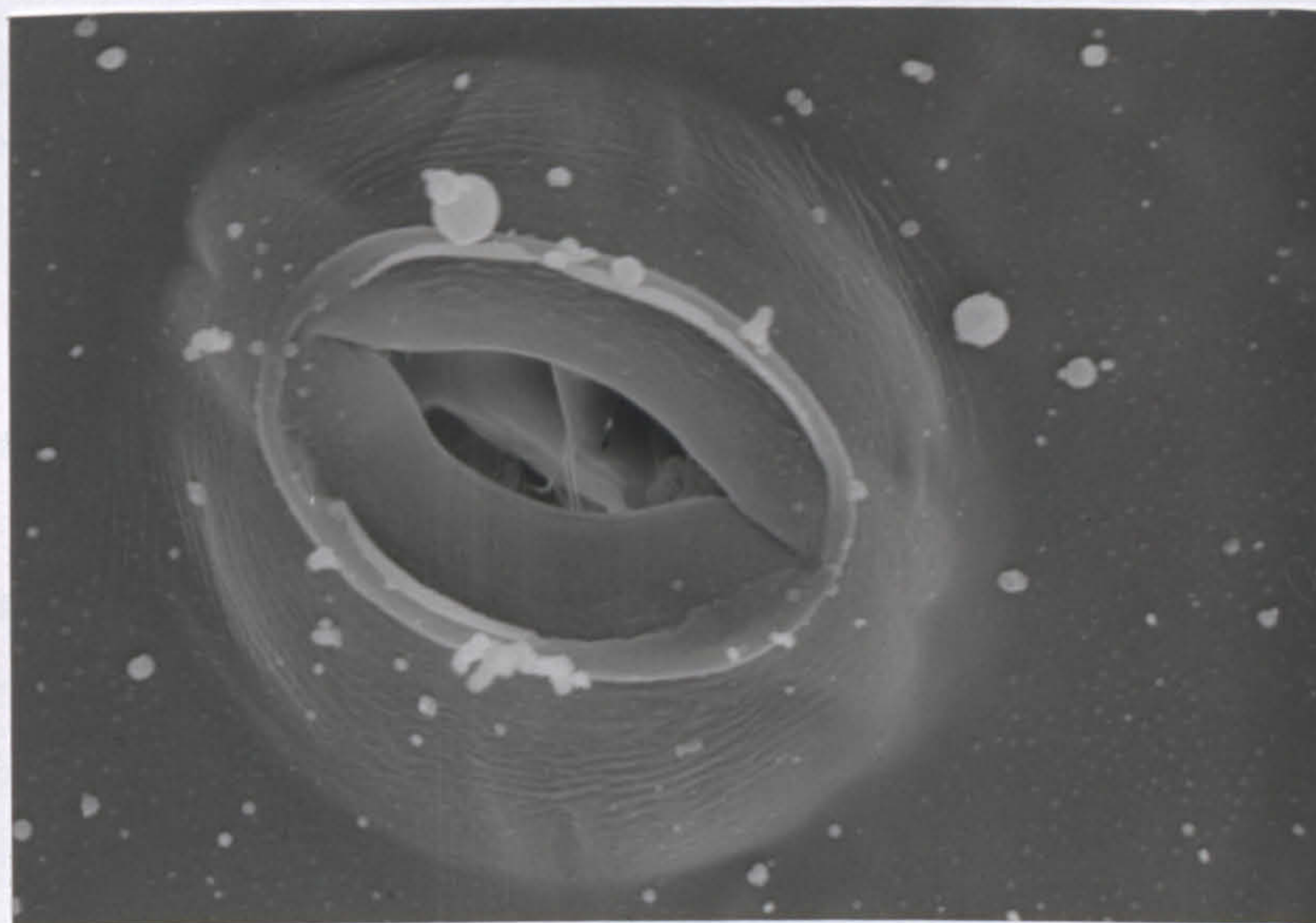
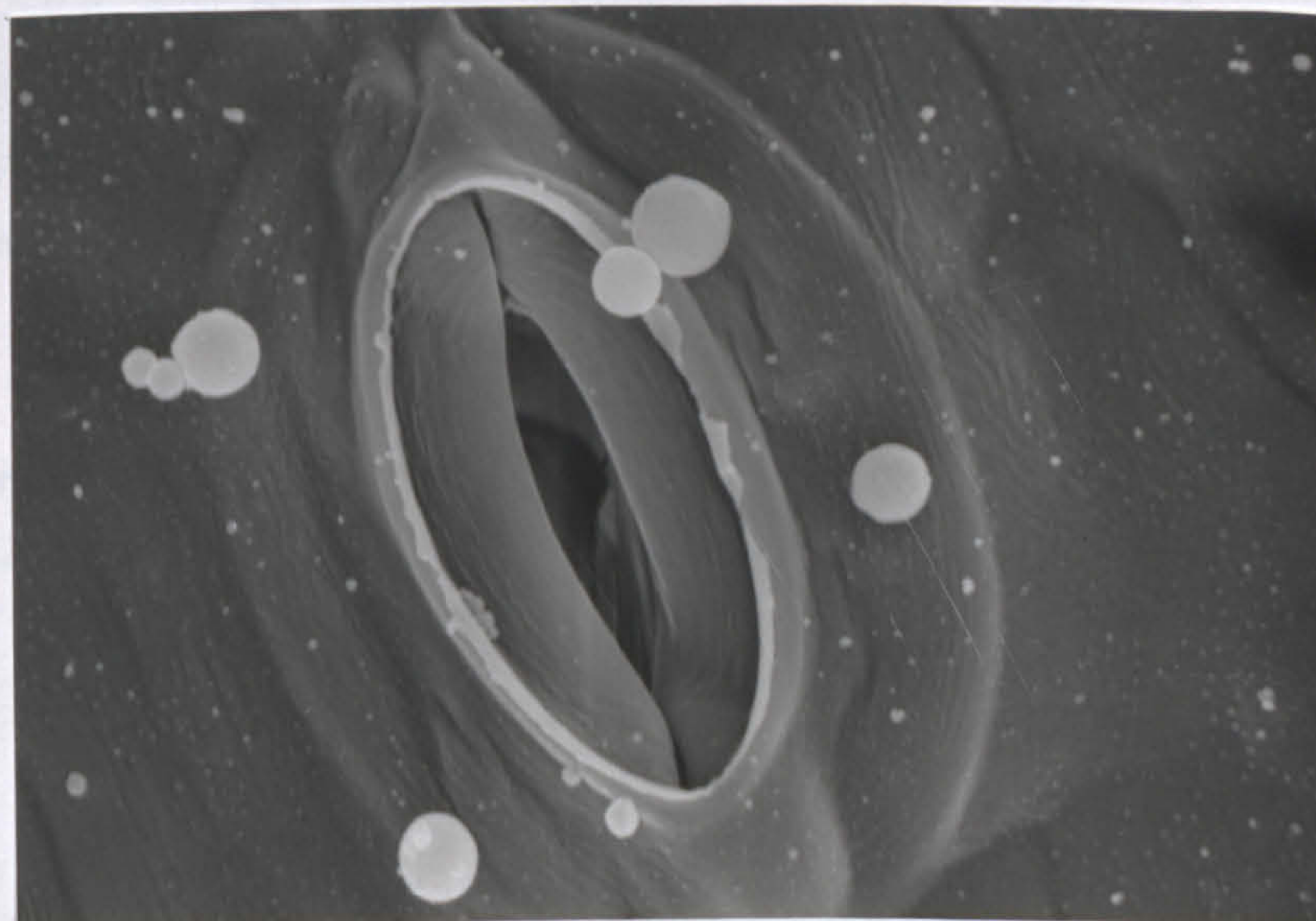


PLATE 10



Data presented in Figure 13 showed that the supply of Ca to shoot-tips and emerging leaves of seedlings which had been subjected to the periodic inversion of tubes alleviated development of STN symptoms on pistachio shoots. Control batches of seedlings cultured in the normal manner showed increasing levels of STN that reached 100% after 28 days of culture.

4.2.4.7 Calcium content of shoot-tips

Because glasshouse-grown seedlings did not develop STN symptoms whereas those grown in vitro did, and in view of the positive effects of liquid feeding on alleviating STN as described above, it was considered of interest to determine whether or not in vitro tissues actually contained less Ca than those produced in vivo. With this in mind, twenty seeds of P. vera were germinated in vitro on filter paper bridges over $1/2$ x MS liquid medium and then half of the cultures were transferred to the glasshouse and potted in a mixture of sand:peat:perlite (1:1:1). Both the in vitro and in vivo batches of plants were grown at 25°C/16h.

Triplicate samples of shoot-tips, each weighing 50 mg (fresh weight) were excised from apparently normal seedlings grown in both conditions and were prepared for Ca analysis using a modification of the basic method developed by Allen (1984). After drying overnight at 85°C, 4-7 mg of each sample were weighed in borosilicate tubes on a microbalance and 0.6 ml of a 5:1 mixture of 72% HClO₄ and 3N HNO₃ was added. Tubes were kept for 20 min on a hotplate heated to 190°C and the Ca level in each sample determined by atomic absorption spectrophotometry.

The calcium contents of seedlings (shoot-tips) grown in glasshouse conditions were more than double those of similar materials grown in vitro, the levels as related to tissue dry weights $\pm$ their SE being 0.39 ± 0.005 and 0.16 ± 0.001 %, respectively.

4.2.4.8 Effects of boron (B) supplementation to the culture medium

As mentioned previously, a deficiency of B has also been suspected to

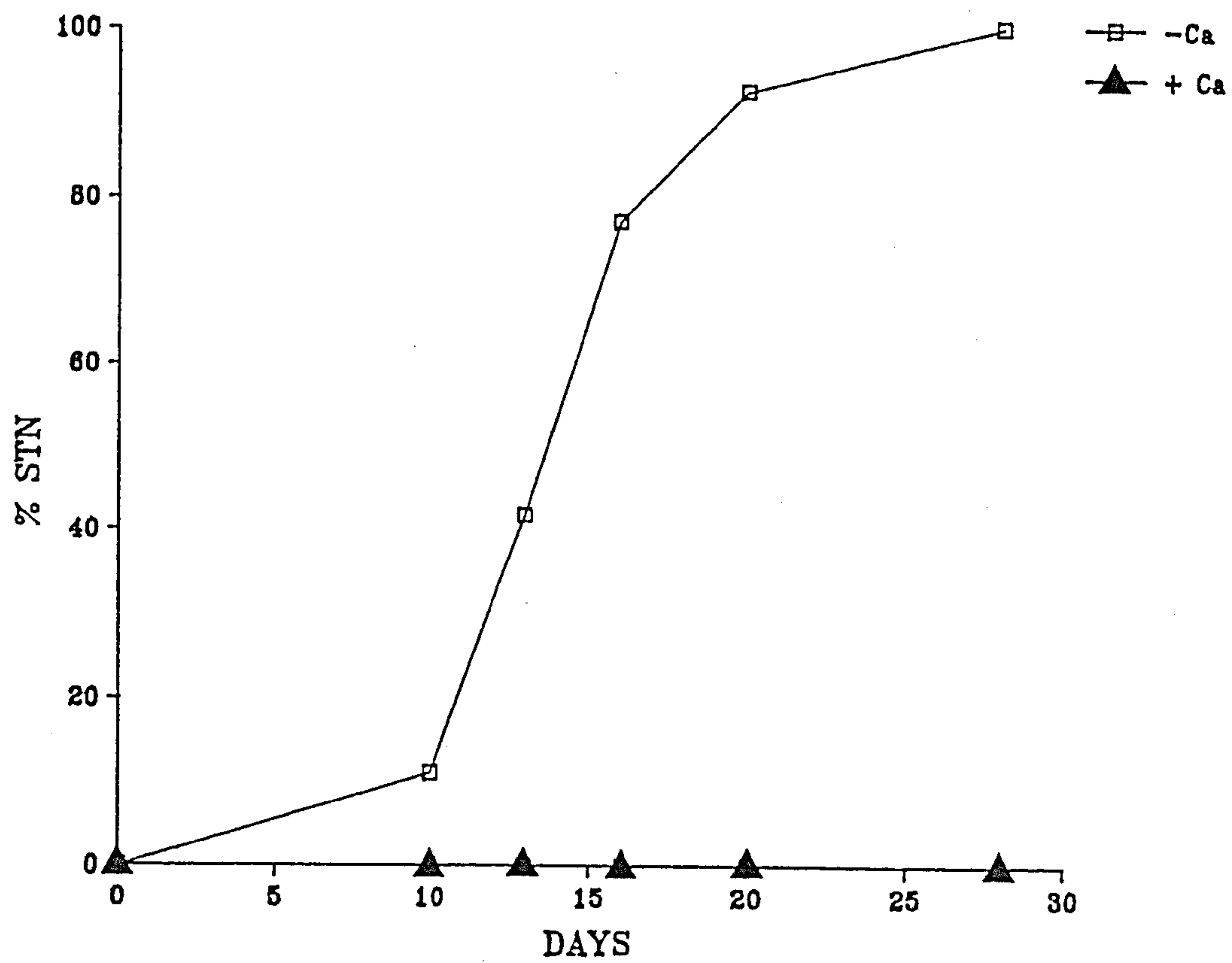


FIGURE 13 : Effects of "feeding" shoot-tips of P. vera seedlings with Ca on STN (n=13).

be involved in the development of STN (Barghchi and Alderson, 1989). B deficiency has been reported to result in the accumulation of phenolic compounds (Dear and Aronoff, 1965). However, Smith and Dugger (1981) obtained no evidence that the accumulation of phenols is important during the early stages of B deficiency in diatom cultures.

In the next experiment, different levels of B supplementation to culture media was undertaken to investigate the effects of B on STN.

Stage II cultures derived from four-year-old P. vera (subcultured x 6) were cultured on MS medium containing 100 μM B as compared with modifications of this medium, made by increasing the level of H_3BO_3 to 200, 500 and 1000 μM . Cultures were incubated for five weeks under standard conditions.

Raising the B level in culture media resulted in a clear reduction in shoot growth and proliferation especially in the case of bud number; the optimal level being 100 μM , i.e., the normal level used in MS medium, and the lowest performances being obtained at 1000 μM level (Table 22).

Concerning STN, symptoms were first observed on media containing 200 μM B. Following five weeks of culture, results were not significantly different and media supplemented with 200 μM remained the most susceptible to development of STN (33.3%) as compared to those shoots cultured on the other media; the latter giving identical results, i.e., 20% (Figure 14).

The level of B used routinely in MS medium (i.e., 100 μM) appears, therefore, to be optimal for growth and development of pistachio shoot cultures and this level of B resulted in relatively low levels of STN.

TABLE 22 : Effects of boron level in modified MS medium on growth and multiplication of four-year-old P. vera derived shoots after five weeks of culture (n=15)

	Boron (μ M)				F test (56 df)	LSD (0.05)
	100	200	500	1000		
% MSF	100.0	100.0	100.0	93.3		
Shoot number ^a	2.5	2.4	2.4	2.0	(P<0.05)	0.36
Total shoot length ^b (mm)	4.0	3.9	3.8	3.5	(P<0.05)	0.37
Total bud number ^a	6.9	6.2	5.9	4.9	(P<0.001)	0.91

a SQR transformed data
b Log_e transformed data

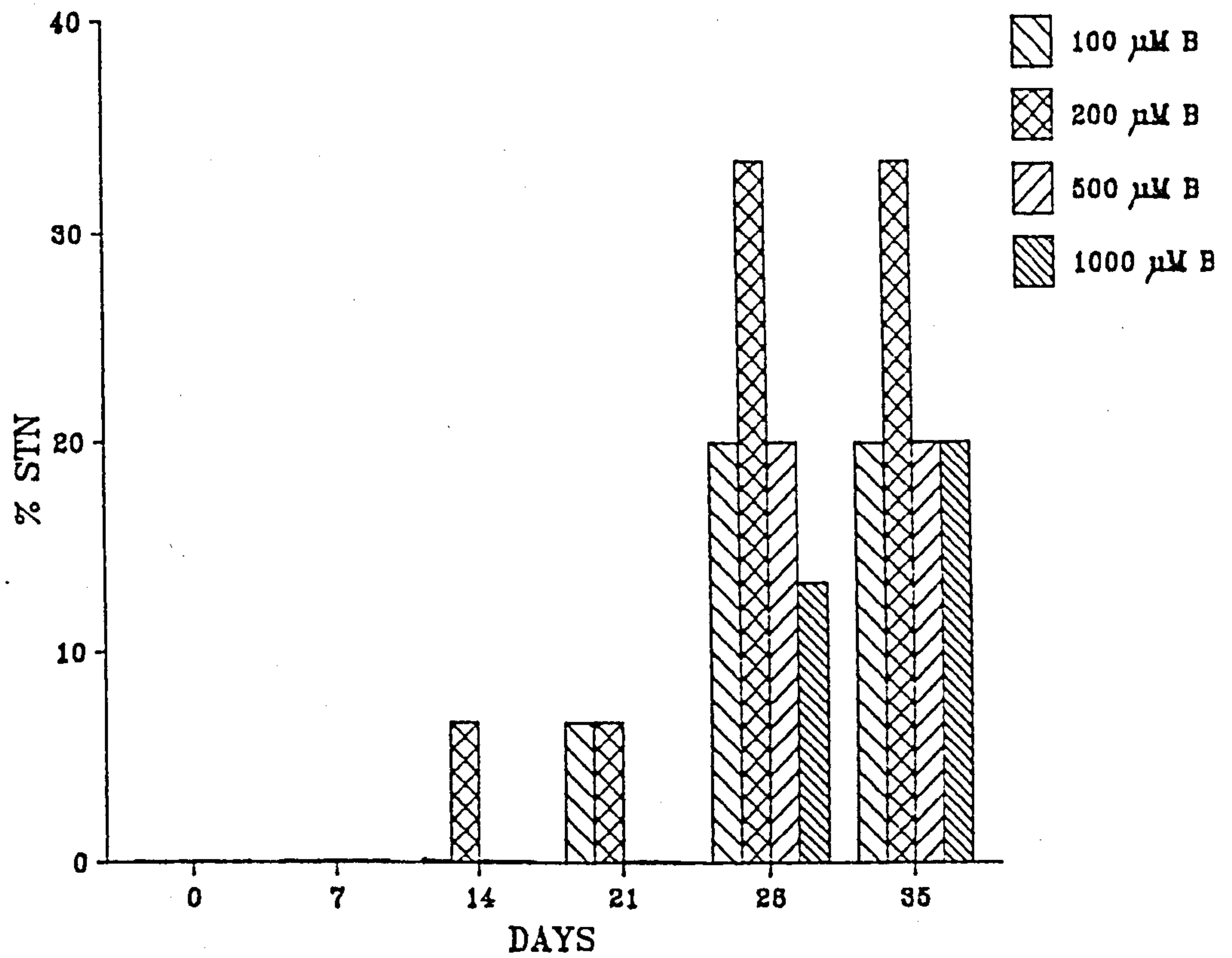


FIGURE 14 : Development of STN in four-year-old *P. vera* derived microshoots incubated at 25°C/16h on media containing increasing levels of B (n=15).
 χ^2_{test} (3 df) : ($P > 0.05$).

4.2.5 Discussion

4.2.5.1 Effects of culture media

The composition of the culture medium is obviously an important factor for achieving successful propagation of plants in vitro. In the current experiments, MS medium proved to be superior for pistachio (Section 4.2.1). Low salt containing media did not support adequate growth of P. vera shoots. The current results contradict those of Barghchi (1985a) who found no differences between the performances of mature P. vera explants when cultured on either MS or WPM media. The differences in performance of shoot cultures which were observed in the current work raised the question as to which factor(s) in MS supports good shoot growth and proliferation. A comparison of the ionic strengths of the tested media showed that MS has a relatively high mineral salt concentration as compared to the WPM, A and K media. Since the level of macronutrients is the major difference between MS and the other media, it was suspected that the relative nitrogen levels in these media might have been the reason for the major differences in growth performances observed; these levels being approximately 18-25% higher in MS as compared to the other formula tested. This seems to indicate that nitrogen is essential for optimum in vitro growth of pistachio. In the case of chestnut, Vieitez et al., (1986) stressed the importance of ammonium ions for optimal growth in vitro as long as its concentration was lower than that present in MS. Moreover, in some clones of Populus, it was found that supplementing WPM with NH_4NO_3 to levels present in MS improved growth and that this growth approached that obtainable on MS (McCown and Sellmer, 1987). Similar trends were reported for shoot cultures of paradox walnut rootstocks; optimal shoot growth responses being obtained with 17.7 mM NH_4NO_3 (Driver and Kuniyuki, 1984).

Although MS medium proved superior when compared with the three other formulae in the present work, it induced more callus and more faintly chlorotic leaves in pistachio. Since MS medium has a relatively high level of salts, Anderson (1980) reported that a general salt toxicity had been responsible for the development of chlorotic shoots of raspberry, since this symptom tended to develop in shoots grown on MS medium rather than in those grown on the A formula. Moreover, since

leaves showing faint chlorosis tended to increase with the raised levels of callus production (which was apparently media-dependent), the basal callus might have acted as a sink or storage tissue for nutrients and/or hormones thus depriving the shoots of essential elements of their growth and development. Iron might be one of these essential elements. For instance, half the iron is reported to be precipitated by phosphate in the MS medium (Dalton et al., 1983), and the use of FeEDTA has been reported to result in chlorotic shoots of hazelnut cultures (Al Kai et al., 1984).

From the results of the current investigations, it was concluded that although the MS medium generally supported the best growth of pistachio in vitro in comparison with other media formulations used widely for tissue culture of tree species, it was not necessarily optimal for shoot multiplication phases of P. vera. Further work needs to be carried out to develop a more suitable growth medium for pistachio shoot culture.

4.2.5.2 Effects of cytokinins

Different responses to BAP were obtained with P. vera (Section 4.2.2.1a) and P. atlantica (Section 4.2.2.1b) seedling materials. In the former species, 4 mg l⁻¹ BAP gave better shoot proliferation than the other tested levels, whereas in the latter species, lower levels (1 mg l⁻¹) of BAP were effective for both shoot proliferation and growth. These results were in agreement with those previously described by Barghchi and Alderson (1983) and Martinelli (1988) for P. vera and P. atlantica seedling materials, respectively. In the former reported work, 4 mg l⁻¹ BAP was found optimal as compared with 0.7 mg l⁻¹ BAP in the latter.

In the case of four-year-old P. vera material (Section 4.2.2.1c), the optimal level of BAP for shoot proliferation and growth seemed to be similar to that obtained with seedling material, i.e., 4 mg l⁻¹. However, at this level over half the cultures showed vitrification symptoms. Since the performance on media supplemented with 3 mg l⁻¹ was not significantly different from that on 4 mg l⁻¹ BAP, this level which induced vitrification in only 18.2% of cultures was considered as more

appropriate for optimal shoot development. Levels of 2 and 3 mg l⁻¹ have been reported suitable for one- to four-year-old (Martinelli, 1988) and two- month-old (Marco-Antonio, 1984) P. vera derived materials, respectively. Different cultivars of P. vera might have responded differently to plant growth regulators as confirmed by Barghchi and Alderson (1989) or, the response might have been age-dependent. It was of interest that responsiveness of four-year-old shoot-derived cultures approached that of seedlings. Since the former type of material was subcultured X3 before it was used in BAP studies, it may have been that some type of acclimatisation of mature material to in vitro conditions may have occurred. A possible "conditioning" of one- to two-year-old pistachio material following several subcultures has also been reported by Barghchi and Alderson (1985). Such "conditioning" may be similar to the apparent rejuvenation observed and described by other workers in mature tree tissues and materials following their passage through several subcultures of micropropagation (e.g., apple shoot cultures as described by Webster and Jones, 1989).

The use of optimal levels of BAP for shoot multiplication produced dwarfed shoots bearing leaves of very small size and showing a rosette type of growth (clusters). Similar features have been observed in chestnut when grown on media supplemented with 1-2 mg l⁻¹ BAP (Vieitez and Vieitez, 1983). The use of 2iP in the current work on P. vera material (Section 4.2.2.2) clearly improved shoot growth resulting in taller shoots bearing more normal larger leaves. However, when this cytokinin was used alone it supported little shoot proliferation. A real improvement in shoot growth without significantly affecting shoot proliferation was possible when a mixture of BAP (1.5 mg l⁻¹) and 2iP (0.5 mg l⁻¹) was used, thus taking advantage of the characteristics of both cytokinins. Anderson (1983) reported that combinations of 2 mg l⁻¹ BAP and 1 mg l⁻¹ 2iP were optimal for shoot proliferation in filbert cultures. The effectiveness of BAP in proliferation and of 2iP in stimulating growth also supported the results obtained on apple shoot cultures by Landergan and Janick (1980).

In conclusion, the results of the experiments concerning the levels of cytokinins used in Stage II of pistachio micropropagation indicated that levels of 3-4 mg l⁻¹ BAP would seem optimal for proliferation of

P. vera material. Supplementing media with mixtures of BAP with 2iP was beneficial for obtaining satisfactory shoot proliferation combined with improved shoot qualities. Lower levels of BAP proved better for P. atlantica seedling material with optimal responses being obtained at 1 mg l⁻¹.

4.2.5.3 Effects of temperature/photoperiod

In the present investigation, shoot growth and proliferation of Stage II cultures (four-year-old P. vera material) incubated under 16h photoperiod were significantly higher at 25°C than at 19°C (Section 4.2.3). By contrast, Barghchi (1985b) reported that although 25°C was better than 20°C, growth and proliferation of P. vera seedling material were not significantly different from those obtained at 20°C. The fact that the pistachio materials used by that worker were of a different cultivar and of a different age (four-year-old vs seedlings) and that different light and cultural conditions were used in the two studies, it was difficult to assess any differences between the results of the current work and those of Barghchi (1985b) especially since Hughes (1981) reported that the temperature range which would allow a normal functioning of plants varies markedly with different species and cultivars.

The results obtained in the current study indicated that incubation of pistachio shoot cultures at 29°C/12h improved shoot growth and proliferation when compared with 25°C/16h, though not significantly except in the case of the fresh weight. Using data collected from different sources, Chalupa (1987) reported that tree species growing in warm regions are incubated under relatively high temperatures, i.e., 25-27°C, while species of subarctic regions were grown at lower temperatures, i.e., 20-25°C. Since at 29°C photoperiod was only 12h and vapour pressure deficit (VPD) was higher than at 25°C/16h (Section 4.2.4.5), it is not known if the observed responses were due to a direct effect of temperature, of photoperiod, of VPD or to a combination of any of these factors.

The results obtained suggested that incubating cultures at 29°C/12h photoperiods might provide suitable conditions for a better

proliferation and growth of four-year-old P. vera material, but this needs further investigation with the use of a larger number of cultures for each treatment combination.

4.2.5.4 Investigations on STN

In the current work, STN was a persistent problem and occurred not only in Stage II but also in Stages I and III of in vitro culture of pistachio shoots. Most of the workers who reported the occurrence of STN in in vitro shoot cultures suspected Ca to be the main factor involved in the development of this physiological disorder (e.g., Sha et al., 1985; McCown and Sellmer, 1987). Boron deficiency has also been reported to cause STN (Hoagland, 1933 in Mason and Guttridge, 1974). Recent publications about pistachio micropropagation have suggested that STN is most likely to result from B or Ca deficiencies (Barghchi and Alderson, 1989). Martinelli (person. comm. 1989) suspected physical rather than chemical factors to be primarily responsible for STN.

The sequential development of visible symptoms as detailed in Section 4.2.4.1 was very important and crucial because it facilitated comparisons with other sequences of STN which have already been reported in the literature. The early visible symptoms consisted of pale brown necrosis developing successively at the tips and edges of the youngest leaves prior to the more general development on whole meristem-tips which darken and die back. These symptoms are similar to those reported to occur in deciduous tree fruits and nuts under field conditions when Ca deficiency is known to be manifested (Shear and Faust, 1980). In the case of glasshouse grown plants, similar symptoms and a sequence of symptom development to the ones described in the present work have been reported on Ca-deficient plants, whereas in the case of B-deficient plants, symptoms appear on the lowest and the oldest leaves on plants and symptoms progressed up the plant rather than in a basipetal manner (Robinson et al., 1987). In deciduous trees fruits and nuts, B deficiency tends to appear usually on fruits before it does on vegetative parts in which case leaves become thick, brittle and dark green. These reports seem, therefore, to suggest that the symptoms observed in pistachio propagated in vitro are manifestations

of a Ca- rather than a B-related disorder.

Results of the TEM studies (Section 4.2.4.3) showed that most of the changes in cell structure were similar to those reported previously by Hecht-Buchholz (1979) on STN in potato sprouts grown under high humidity, and to those of Marinos (1962) on the shoot apex of barley plants grown on Ca-deficient culture medium. The finding that the beginning of the stages of cell degeneration which consisted of invagination followed by disintegration of the plasmalemma and accumulation of degenerative vesicles were in total agreement with those of Hecht-Buchholz (1979). As disintegration progressed, the observed degeneration of the cell wall structure also confirmed the results of Marinos (1962). Both workers reported that such structural changes were observed in Ca-deficient cells. The hypothesis formulated by the latter author was that changes in membrane permeability caused by lack of Ca were responsible for the observed rapid cell autolysis. Marinos (1962) stressed that the cytological effects of Ca-deficiency observed in the shoot apex of barley were consistent with the description of the function of Ca in the regulation of fundamental physiological processes (i.e., membrane and cell wall permeabilities in both plant and animal cells). The results of several authors have indicated the primary involvement of Ca ions in maintaining functional structure. Results of the current ultrastructure studies, therefore, seemed to support the suggestions that Ca deficiency is most likely to be responsible for the development of STN in pistachio cultures.

Supplementing media with increased levels of Ca over those normally present in the MS medium (to levels of up to 30 mM) resulted in a significant reduction of STN but did not prevent its presence (Section 4.2.4.2). Barghchi (1986) in Barghchi and Alderson (1989) reported that STN was controlled in P. vera by application of 12-24 mM Ca. Since no details of experiments and data were presented in the above-cited reference, it is not clear whether the symptoms were completely prevented or just reduced and if so, to what extent. It is also not clear which form of Ca was used. In the current work, calcium gluconate which is an organic form of Ca was used effectively to control STN. No modifications of the basal media components were needed therefore, and the increase in inorganic ions (e.g., chloride

and nitrate) which would have resulted from any increase in Ca level (using CaCl_2 or $\text{Ca}(\text{NO}_3)_2$, respectively) was avoided. Addition of up to 30 mM Ca gluconate improved shoot growth and proliferation.

As far as the involvement of boron is concerned, the results obtained (Section 4.2.4.8) showed that increasing levels above $100\mu\text{M}$ (which is normally used in MS medium) appears to inhibit rather than stimulate growth and proliferation of P. vera shoots. This is in agreement with the findings of Barghchi (1986c) in Barghchi and Alderson (1989) who reported a reduction in shoot multiplication following B application, as well as with the results of glasshouse studies carried out on pistachio by Sepaskhah et al. (1988). However, the fact that STN was increased, although non-significantly, when $200\mu\text{M}$ B was added to the culture medium, and that higher levels neither prevented nor reduced STN by 35 days of culture conflicted with the results reported by Barghchi and Alderson (1989). In their work, levels of B higher than $200\mu\text{M}$ apparently controlled STN in P. vera. The results obtained in the current work seem to agree with those of Mason and Guttridge (1974) who found that on NFT-grown strawberry plants, tipburn occurred first sporadically and independently from the level of B in the nutrient solution and it only later developed in plants grown at all levels of B. Noticeably, it even occurred on plants showing B toxicity symptoms. Moreover, B failed to alleviate the tipburn condition in strawberries when applied as aqueous drops directly to buds and emerging leaves while Ca almost prevented its occurrence.

An inefficient distribution of Ca rather than poor Ca uptake in plants is believed to be responsible for Ca-related physiological disorders (Bangerth, 1979) and this would explain why Ca deficiency disorders can frequently occur even on Ca-rich soils (Millaway and Wiersholm, 1979 in Bangerth, 1979). Ca deficiency has been reported to be normally restricted to organs and tissues which possess a low transpiration rate but a high demand for assimilates (Bangerth, 1979). A high relative humidity of the gaseous atmosphere in culture vessels is typical of tissue culture systems, thus resulting in a depression of transpiration activity and possibly in creating the conditions for Ca-related disorders to develop in fast growing shoot-tips. This situation is made even more acute by the fact that pistachio is a very

gypso-calciphilous species as reported by Woodroof (1979) and as such, the contents of Ca tend to be higher in the natural situation in the field in the lower rather than in the upper parts of pistachio plants (Parsa and Wallace, 1980). Estimates of VPD levels are more precise determinants of potential transpiration activities than are RH levels of the gaseous environment surrounding a plant tissue (Kramer and Kozlowski, 1979). When pistachio shoots were grown at either 25°C/16h or 29°C/12h (conditions which resulted in a significant improvement in shoot growth and proliferation), STN levels were 50 and 20%, respectively. Estimates of VPD (Section 4.2.4.5) were higher at 29°C/12h than at 25°C/16h (i.e., 2.3 vs 0.6 mb, respectively). Examinations of stomata on leaves of in vitro pistachio shoots maintained at 29°C/12h showed that stomatal pores were apparently normal and functional in these plants, whereas under 25°C/16h they were large and wide open, and resembled the forms on non-functional stomata typical of vitrified shoots (Ebida, 1988). Holder and Kockshull (1988) have found a correlation between severity of Ca deficiency in tomato plants grown under glasshouse conditions and various humidity treatments. High RH levels have also been reported to reduce the percentage distribution of ^{45}Ca to shoot apices of tomato plants grown in recirculating nutrient solutions (Ehret and Ho, 1986). In the present work sealing of vessels with nescofilm resulted in a quick and intense development of STN (Section 4.2.4.2f). Similar effects have been reported by Sha et al., (1985) with potato shoot cultures. Vessel closure types that allow more air exchange are reported to have reduced STN but at the same time resulted in medium dessication, thus necessitating frequent subcultures (McCown and Sellmer, 1987). Tighter seals might have maintained higher humidity levels within culture vessels, thus creating environmental conditions which inhibited adequate supplies of Ca to the rapidly growing tissues and organs. This is an explanation also put forward by Sha et al., (1985) in their work on STN in potato shoot cultures. Actually, as found in previous experiments of this work, Pistacia seedlings grown in the glasshouse did not develop STN, in contrast with in vitro grown ones, which all showed STN after only four to five weeks of culture; the Ca content of shoot-tips in the former case being more than twice that obtained in the latter (Section 4.2.4.7).

The fact that in the current work a periodic inversion of tubes containing liquid medium supplemented with high levels of Ca (15 mM) completely prevented STN development under conditions where these would normally have arisen (Section 4.2.4.6) suggested that the direct application of Ca to the susceptible emerging leaves/shoot-tips was sufficient to overcome the poor distribution of Ca caused by the low transpiration rates in pistachio shoot cultures due to high humidity within culture vessels. Applications of aqueous solutions of Ca salts to buds and emerging leaves of strawberry plants grown in nutrient solution in glasshouse experiments have been reported to almost completely prevent tipburn in this species (Mason and Guttridge, 1974). Spraying of Ca (Mahanty and Fineman, 1975) or a post-harvest dip in Ca solution (Bangerth, 1973) of apple fruits have been successfully practiced to reduce bitter pit, a Ca-related disorder.

The analysis of data showing the effects of different cultural and environmental factors in STN seems to indicate that the appearance of STN is associated with rapidly growing cultures. This was particularly noticeable at Stage II when optimal levels of BAP were used as opposed to sub- or supraoptimal levels (Section 4.2.4.2c), when BAP was used as compared with 2iP (Section 4.2.4.2b), on phytozel as opposed to agar (Section 4.2.4.2d), and at 25°C and 29°C as opposed to 19°C (Section 4.2.4.2e). In studying the occurrence and cause of STN in potato shoot cultures, Sha et al., (1985) concluded that STN is associated with Ca deficiency in actively growing shoot cultures. In a recent publication, Borkowska and Michalczyk (1989) studying the uptake and translocation of ⁴⁵Ca in sour cherry shoot cultures came to the same conclusion; these workers suggested that the rates of growth in rapidly developing plantlets were considerably faster than the comparable rates of Ca accumulation, and this situation resulted in lower relative Ca contents in shoots than in those situations where Ca uptake and distribution proceeded in pace with the growth and development of shoots.

Concerning the cytokinin effect mentioned above, supplementing media with low levels of BAP at Stages IB and II (Section 4.2.4.2c) reduced STN levels. The movement of ⁴⁵Ca from the roots into developing leaves of apple seedlings has been reported to occur following foliar sprays

of BAP and kinetin (Shear and Faust, 1970). Furthermore, increases in membrane associated Ca were detected in Funaria during bud formation and this indicated a likelihood of a localized rise in intracellular-free Ca levels brought about by cytokinin action (Saunders and Helper, 1981).

Increasing the strength of the MS medium at Stage IA (Section 4.2.4.2a) resulted in an increased development of STN. Tomato plants grown in recirculating nutrient solutions held at different electrical conductivities have been reported to result in reduced uptake of both water and ^{45}Ca not only under conditions of high RH, but also under conditions of high salinity, thereby inducing the development of Ca related physiological disorders in the plants (Ehret and Ho, 1986; Ho, 1988).

In contrast with the finding of Barghchi and Alderson (1985) who reported that root development on P. vera shoots (rooted in vitro) prevented the development of STN, results of the present study (Section 4.2.4.2g) showed that the rooting of the cv. Mateur shoots only reduced STN and did not prevent the development of STN symptoms. Similar results have recently been reported in chestnut and oak shoot cultures (Vieitez et al., 1989). The latter authors reduced STN occurrence by supplementing rooting media with low levels of BAP. However, not surprisingly, this practice considerably reduced rooting in vitro. These workers suggested that endogenous cytokinin production or the improved nutrient uptake caused by the presence of roots might have been primary factors involved in preventing STN. They also formulated the hypothesis of auxin involvement in the process since a quick and intense development of STN was observed when shoots were treated with IBA. Actually, Barghchi and Alderson (1985) also found that adding as little as 0.25 NAA to the multiplication medium containing BAP increased STN of P. vera shoots propagated in vitro. These latter results suggested that NAA might have altered the availability and the production of endogenous cytokinin which have been reported to inhibit the occurrence of STN. Using ^{45}Ca , Borkowska and Michalczyk (1989) recently demonstrated that the presence of roots resulted in a higher accumulation of ^{45}Ca in leaves of cherry shoots grown in vitro. These workers suggested that root formation changed the translocation pathway

of Ca from xylem to phloem, which is reported to be independent of the transpiration rate and consequently more effective under in vitro conditions.

Other genotypic and physiological factors could have played significant roles also in the manifestation of STN in pistachio shoot cultures. In the current investigation, a marked difference in susceptibility of shoot cultures to develop STN was found not only between different species, but also within the same species. In the former case, P. vera was more sensitive than P. atlantica (Section 4.2.4.2h) and this was confirmed in observations on cultures of Stage I and II. Martinelli (person. comm., 1989) has also reported similar trends in STN susceptibility of these two species particularly in Stage II. The capacity to immobilize Ca has been reported to depend upon species and cultivars (Parsa and Wallace, 1980; Gallaher, 1975), and the latter author suspected Ca deficiency to be affected by this capacity. Differences were also observed in levels of STN symptoms between four-year-old-derived shoots and seedling materials of P. vera at Stage II; the former showing greater levels than the latter (Section 4.2.4.2i). Interestingly, translocation of ^{45}Ca has been shown to be greater in apple seedlings showing juvenile leaf characteristics than in those of the similar age but without juvenile characters (Shear and Faust, 1970).

Noticeably, apical dominance appeared to be stronger in P. vera than in P. atlantica seedlings (Stage IB Section 4.1.2), and in four-year-old as opposed to P. vera seedling-derived shoots (Stage II). Synthesis of IAA in the apical parts of shoots, particularly in the young expanding leaves, and its transport down the stem has been reported to be the basic component of the mechanism of apical dominance (Wareing and Phillips, 1981). A close relationship between the mechanism of auxin action and Ca has been reported by several authors such as Evans (1985) and Cunningham and Hall, (1986). Quinlan and Preston (1973) reported that selective disturbance of shoot-tips in apple trees might be an alternative to auxin applications, presumably because of the elimination or the reduction of any strong competition of shoot-tips for Ca, which might be due to their inherently high auxin production. The high levels of auxin likely to be present in the apical bud might have been increased to supraoptimal levels because of phenols.

Compounds such as chlorogenic acid have already been reported to inhibit IAA oxidase (Lee et al., 1982), a phenomenon which has been reported to occur in pistachio cuttings, presumably because of high levels of phenolics (Marco- Antonio, 1984). Noticeably, P. vera seedling material produced more phenolics than did P. atlantica and this was also confirmed during the TEM investigations (Section 4.2.4.3) since pretreatment of tissues with antibrowning compounds like AA or caffeine was necessary in the case of the former species in order to allow adequate visualization of organelles and inclusions within the cytoplasm of cells. In contrast, specimens of P. atlantica were easily examined and did not require any such pretreatment. In lettuce, for instance, the most susceptible cultivars to tipburn are reported to have the highest levels of chlorogenic acid (Collier et al., 1979 in Bangerth, 1979). According to Crisp et al. (1976), it would appear that limited supplies of Ca during auxin-mediated tissue extension (especially for maintaining the constitution of the cell walls) would result in cell damage and necrosis. The obvious effects of high humidities within tissue culture vessels on transpiration and consequently on Ca uptake and translocation, and the stimulation of STN by auxin (reported for pistachio, chestnut and oak grown in vitro) have already been discussed above.

From the results obtained in this study STN would appear as a Ca-related disorder associated with actively growing shoot cultures and low VPD conditions. The strong apical dominance and the presence of phenolic compounds characterizing pistachio might have made this problem more acute. A direct application of Ca to the susceptible shoot-tips proved appropriate in overcoming the induction of STN symptoms on fast growing, low transpiring cultures of pistachio at Stage I. This approach might also be applicable to Stage II cultures and this aspect needs further investigations in the future.

4.3 STAGE III - ROOTING

In vitro rooting of P. vera has been achieved for shoot cultures derived from up to two-year-old seedling material (Barghchi and Alderson, 1985, 1989), and from shoot cultures derived from one- to four-year-old grafted plants (Martinelli, 1988). Rooting was more successful in the former as compared to the latter types of material. In vitro rooting of P. atlantica has also been reported using explants taken from a few months to four-year-old materials but no data were presented (Martinelli, 1988).

In the present studies, in vitro rooting experiments were carried out using seedling materials of P. vera and P. atlantica, as well as shoot materials taken from four-year-old grafted P. vera cv. Mateur since the former and the latter types of material are the main rootstocks and cultivars, respectively, cultivated in Morocco. The effects of IBA, subculture number and micronutrient status of media treatments were investigated. In vitro rooting of pistachio shoots following a quick dip in high concentrations of IBA was also attempted.

4.3.1 Seedling Material

4.3.1.1 Effects of subculture number on multiplication medium

(Stage II) and IBA treatments on rooting of in vitro shoots

Several factors including treatments with growth regulators in general, and BAP in particular (Nemeth, 1986; Wainwright, 1987) and of subculture number (Webster and Jones, 1989) during shoot multiplication (Stage II) have been reported to affect rooting response in vitro.

In the following experiments, the effects of subculture number during Stage II on rooting on media with different levels of IBA was investigated using both P. vera and P. atlantica seedling materials.

a) P. vera

Nodal segments were excised from in vitro grown seedlings as well as from materials obtained from shoots after one or two subcultures on multiplication medium containing 4 mg l^{-1} BAP, and were induced to root

on $1/2$ X MS media, either hormone-free or supplemented with 0.5, 2.5 or 3 mg l^{-1} IBA. Cultures were incubated for one week in darkness at 25°C before being transferred to the light (16h). Shoots were subcultured on auxin-free medium once the root primordia were visible, thus following the procedure used by Barghchi and Alderson (1983).

Shoots subcultured once on multiplication medium responded better to IBA treatments than did those which had been directly obtained from seedlings or those which had been subcultured twice on multiplication medium; the highest percentage of rooting (75%) was obtained at 2.5 mg l^{-1} IBA. No rooting was ever observed when the hormone-free medium was used (Figure 15). Concerning root number, the results obtained provided evidence of a real effect of subculture and of IBA on rooting. Again, shoots subcultured once and treated with 2.5 mg l^{-1} IBA produced optimal root number per shoot (Table 23). No interactions seemed to exist between the subculture number at Stage II and IBA level on rooting of in vitro shoots.

b) P. atlantica

Explants obtained from in vitro grown seedlings and from cultures subcultured once on Stage II medium containing 1 mg l^{-1} BAP, were transferred to Stage III media ($1/2$ x MS) which were either hormone-free or supplemented with 0.5, 1, 1.5, 2, 2.5 or 3 mg l^{-1} IBA. Otherwise, culture conditions were similar to those described for P. vera.

Rooting responses followed trends similar to those obtained with P. vera; 87.5% rooting was obtained on shoots derived from Stage II cultures as compared with 62.5% observed on shoots directly excised from germinating seedlings. The optimal responses were obtained at 1 and 2 mg l^{-1} , respectively (Figure 16). IBA was the only factor with a strong effect on root number with the above levels being also optimal for the two types of materials (Table 24).

4.3.1.2 Optimal IBA level for rooting P. vera derived shoots

In the previous experiments, rooting performances of P. vera seedling

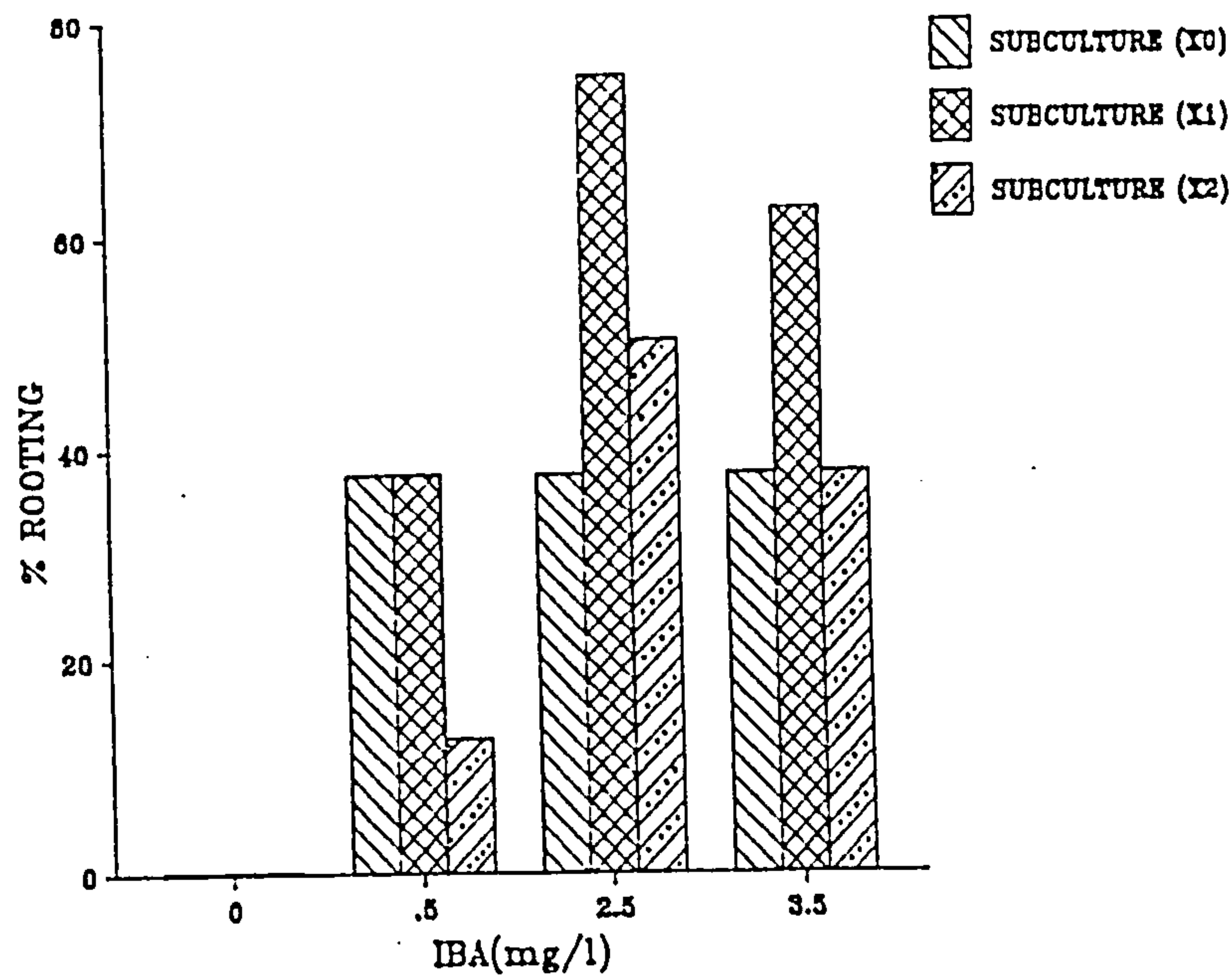


FIGURE 15 : Effects of Stage II subculture number and IBA treatments (Stage III) on percentage rooting of *P. vera* seedling derived microshoots (n=16).
 χ^2 test (subculture x 1) (3 df) : (P<0.001).

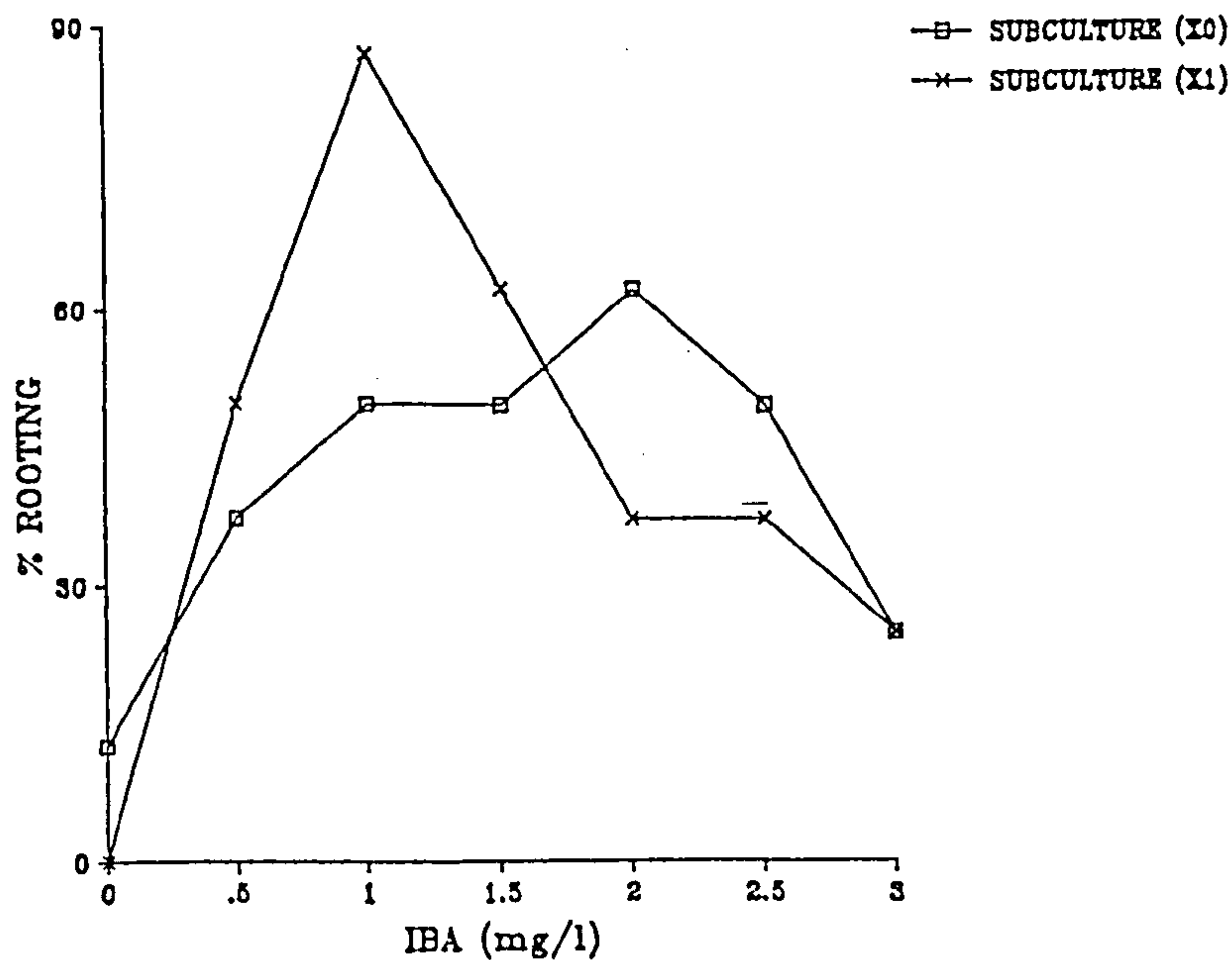


FIGURE 16 : Effects of Stage II subculture number and IBA treatments (Stage III) on percentage rooting of *P. atlantica* seedling derived microshoots (n=8).

TABLE 24 : Effects of subculture during Stage II and IBA levels used at Stage III on mean root number of P. atlantica seedling material after 21 days of culture (n=8)

Subculture		IBA (mg l ⁻¹)							Mean (subculture)
		Number	0.0	0.5	1.0	1.5	2.0	2.5	3.0
Root number per shoot ^a	x 0		0.6	0.9	0.9	1.0	1.3	0.9	0.7
	x 1		0.6	1.1	1.5	1.2	0.9	0.9	0.7
	Mean (IBA)		0.6	1.0	1.2	1.1	1.1	0.9	0.7
			F test						
			(98 df)						
			LSD						
			(0.05)						
Main effect subculture			(P>0.05)						
Main effect IBA			(P<0.01)						
Interaction subculture x IBA			(P>0.05)						

^a SQR (n+0.5) transformed data

materials were better on 2.5 mg l⁻¹ than on any of the other tested IBA levels (i.e., 0.5 and 3.5). In the current experiment, concentrations of 1.5, 2, 2.5 and 3 mg l⁻¹ were used therefore, in order to select the optimal level for rooting in vitro. Culture conditions were as previously described (Section 4.3.1.1).

Optimal rooting percentage and mean root number per shoot were obtained on media supplemented with 2 mg l⁻¹. However, these obtained levels were not significantly different from those achieved with 2.5 mg l⁻¹ IBA (Table 25).

TABLE 25 : Effects of IBA levels on rooting of in vitro seedling materials after 21 days of culture (n=16)

	IBA (mg l ⁻¹)				F _{test} (60 df)	LSD (0.05)	X ² _{test} (4 df)
	1.5	2.0	2.5	3.0			
% Rooting	12.5	75.0	50.0	37.5			(P<0.05)
Root No per shoot ^a	0.9	1.6	1.4	1.3	(P<0.05)	0.44	

^a SQR (n+0.5) transformed data.

4.3.1.3 Effects of micronutrients

Since MS medium used in the current work was factory-prepared as a complete medium and because Barghchi and Alderson (1983) found that rooting of seedling P. vera derived shoots was improved by reducing the macronutrients to half their normal strength in MS medium, modifications were made to rooting media using stock solutions in order to study the effects of micronutrients on rooting of P. vera (cv. Mateur) seedling materials (subcultured x 1). Performances on 1/2 x MS medium were, therefore, compared with those obtained on 1/2 x MS macronutrients; vitamins being maintained at half strength the usual levels of the MS medium. Root induction was performed on Stage III media containing 2 mg l⁻¹ IBA and culture conditions used were as in the previous rooting experiments.

Results (Table 26) showed that rooting was better achieved on medium containing full-strength micronutrients than on half-strength

micronutrients. However, this difference was not significant. Noticeably, roots appeared first on the full-strength medium after five days of culture whereas on half-strength medium the first signs of rooting occurred two days later.

TABLE 26 : Effects of micronutrients on rooting of P. vera seedling materials after 21 days of culture (n=12)

	Micronutrient strength ^a		F _{test} (22 df)	LSD (0.05)	X ² _{test} (1 df)
	x 1/2	x 1			
% Rooting	66.7	83.3			(P>0.05)
Root No per shoot	2.0	3.3	(P>0.05)	1.91	

^a Both media supplemented with 2 mg l⁻¹ IBA.

4.3.2 Four-year-old plant material

In vitro propagated shoots of mature Pistacia have been reported to be difficult to root. This was reported for P. terebinthus (Pontikis, 1985) and P. vera (Martinelli, 1988) using the conventional method of inducing rooting on medium containing relatively low levels of auxin before subculturing on hormone-free medium. Dipping the basal ends of microcuttings in concentrated auxin solutions for a short time (several seconds) is increasingly used to root microshoots and cuttings of recalcitrant tree species. Such an approach has been used either in vitro (Vieitez et al., 1986; Qi-Guang et al., 1986; Chauvin and Salesses, 1988) or in vivo (Driver and Kuniyuki, 1984; Ponchia and Howard, 1988).

In the following experiment the newer approach was attempted on four-year-old P. vera material. The basal cut ends of Stage II-derived cultures (subcultured x 6) were dipped to a depth of c. 5mm for 10s in a filter-sterilized 20% ethanolic solution of IBA; the concentrations used being 100, 500 and 1,000 mg l⁻¹. The control consisted of microcuttings treated with 20% ethanol. A higher level of IBA (5,000 mg l⁻¹) was also prepared, but a rapid crystallization of this solution occurred once the solution was exposed inside a laminar flow cabinet. Therefore, this treatment was discarded. Treated shoots were

transferred to $1/2$ x MS macronutrient medium free from auxins and solidified with agar. Cultures were kept at 25°C under 16h photoperiod.

After five weeks of culture, a real improvement in rooting percentage was obtained with increasing levels of IBA; treatments with 500 and 1,000 mg l^{-1} IBA being the only ones inducing rooting at 25 and 50%, respectively. No significant differences in root number were observed between the two treatments (Table 27).

TABLE 27 : Rooting responses of four-year-old Stage II P. vera shoots rooted in vitro following a 10 s dip in IBA solution (n=12)

	IBA (mg l^{-1})				F _{test} ^a (22 df)	LSD (0.05)
	0	100	500	1000		
% Rooting	0.0	0.0	25.0	50.0		
Root No per shoot ^b	0.0	0.0	0.8	1.2	(P>0.05)	0.41

^a Comparison between 500 and 1000 mg l^{-1} IBA.

^b SQR (n+0.5) transformed data taken at five weeks after treatment.

4.3.3 Discussion

In the current work, seedling material of both P. vera and P. atlantica were readily rooted in vitro following the procedure described by Barghchi and Alderson (1983). The rooting percentages achieved were 83.3 and 87.5% with an average of 4 and 3 roots per rooted shoots of P. vera and P. atlantica, respectively. The latter authors also easily rooted P. vera seedling material and Martinelli (1988) reported that seedling explants of P. atlantica showed a good potential for rooting in vitro.

Although the numbers of Stage II subcultures were limited because of difficulties in obtaining suitable shoots for rooting without going through an elongation phase, it would appear that subculturing affects rooting response. Stage II derived cultures which had been subcultured only once showed the best potential by far for rooting as compared with other materials obtained either after two subcultures at Stage II, or directly from stage IA (i.e., excised from seedlings germinated in vitro). These results suggested that explants taken directly from seedlings had not been conditioned sufficiently to in vitro conditions. Barghchi and Alderson (1985) reported also poor rooting when initial explants from P. vera seedlings were used. Alternatively, Stage IA shoots which have been subcultured more than once at Stage II medium with a high cytokinin level (4 mg l^{-1} BAP) performed less well (i.e., 50%). The effects of the conditions during Stage II cultures immediately preceding Stage II have also been reported to be critical to rooting responses. For example, Wainwright (1987) found that rooting percentage of Tayberrry microshoots decreased with use of increasing concentrations of cytokinin in Stage II shoot multiplication media. Moreover, cytokinin has been reported to generally inhibit root formation (Gaspar and Coumans, 1987) so these observations and results were not surprising.

In the current work, optimal levels of IBA for rooting of P. vera and P. atlantica seedling shoot materials were, respectively, 2 and 1 mg l^{-1} . This suggests that there is a requirement for a higher level of plant growth regulators by P. vera than by P. atlantica. Interestingly, these type of differences in growth regulator

responsiveness were also reflected in the cytokinin levels required for shoot multiplication (Section 4.2.2.1). The use of 2 mg l^{-1} IBA for inducing roots on microshoots of P. vera approaches the recommendation by Barghchi and Alderson (1983) in their work on P. vera seedling materials (i.e., 2.5 mg l^{-1}).

The improvements obtained in rooting percentages (from 67 to 83%) and in the number of mean roots produced per rooted microshoot (i.e., from 3 to 4) which were produced when medium containing full-strength MS micronutrients were used instead of half-strength micronutrients may have been due to the promoting effects on rooting exerted by micronutrients. Inhibitory effects on rooting have been reported by Barghchi and Alderson (1983) for pistachio when complete MS medium was diluted to different levels ($\times 1/2$, $\times 1/4$). These authors suspected that a deficiency of either micronutrients or vitamins may have been responsible for the decreased rooting observed.

Rooting of mature P. vera material was achieved using the "quick" auxin-dip approach. Up to 50% of the shoots rooted with a mean of three roots per rooted shoot after five weeks of culture. This best rooting level was achieved by dipping the bases of shoots for 10 s in $1,000 \text{ mg l}^{-1}$ IBA. P. vera cultivars obtained from one- to four-year-old grafted plants has previously given 30-40% rooting in vitro (Martinelli, 1988). However, no details of the method and conditions used nor the history of cultures were reported. The rooting responses of the mature materials used in the current study compared with those achieved with seedling material directly established in vivo after a "quick" dip in IBA solutions (Stage IV, Section 4.4.2) confirm the difficulties usually encountered in rooting cuttings of mature Pistacia material (Assaf, 1971; Joley and Opitz, 1971; Al Barazi and Schwabe, 1982). The latter authors obtained 85% rooting on mature softwood cuttings of P. vera under mist but only after treatment with an extremely high level of IBA ($35,000 \text{ mg l}^{-1}$).

In conclusion, P. vera and P. atlantica seedling materials could be rooted easily in vitro with the latter species responding to lower IBA levels than the former one. The conditions of culture immediately preceding the rooting stage as well as the mineral composition of the

rooting medium appeared to be important influences on the rooting responses obtained. Finally, current results confirmed the recalcitrant character of mature Pistacia material at the in vitro level.

4.4 STAGE IV - WEANING

Considerable care is reported to be needed during transplanting and acclimatization of in vitro propagated shoots (Conner and Thomas, 1981; Dunstan and Turner, 1984). An alternative to rooting shoots in vitro and then transferring them to glasshouse or field conditions is to root tissue culture-derived shoots directly in vivo. This procedure has the advantage of lowering plant production costs (Debergh and Maene, 1981).

In the following experiments which were carried out in the Wye College glasshouse in the months of October and November 1989, acclimatization of in vitro as well as in vivo rooted P. vera seedling material was investigated.

4.4.1 In vitro Rooted Shoots

In vitro rooted shoots of P. vera seedling material (Section 4.3.1) were treated as previously described in Section 3.4.

After five weeks from transplanting propagules to compost mixture, shoots established well and a level of 81.8% plantlet survival was achieved.

4.4.2 Direct Rooting In vivo

Explants of in vitro seedlings, instead of being rooted in vitro, were directly rooted in vivo. The basal cut ends of explants were dipped in IBA solutions as specified in Section 4.3.2 except that the time of treatment was reduced to 5 s instead of 10 s. Microcuttings were directly potted in a mixture of peat and grit (1:1, v:v) and were treated as described in Section 3.4.

Rooting was induced at all the tested IBA levels with 100% rooting being obtained at 500 and 1,000 mg l⁻¹. Lower rooting (77.8%) was obtained at 100 mg l⁻¹. No rooting was observed when shoots were not treated with IBA (Table 28). No significant differences were observed between the mean numbers of roots produced per microshoot, these increasing with the raising of IBA concentration from 2.3 to 4.4

roots per shoot.

TABLE 28 : Rooting responses of P. vera seedling derived shoot cultures directly rooted in vivo following a 5 s dip in IBA solution (n=9)

	IBA (mg l ⁻¹)				F _{test} ^a (22 df)	LSD (0.05)
	0	100	500	1000		
% Rooting ^b	0.0	77.8	100	100		
Root No per shoot ^b	0.0	2.3	3.0	4.4	(P>0.05)	2.40

^a Comparison excluding the control treatment.

^b At five weeks after treatment.

4.4.3 Discussion

Acclimatization of in vitro rooted shoots of P. vera seedling material was readily achieved in the Wye College glasshouse with 81.8% survival obtained. Similar results have been reported by Barghchi and Alderson (1985) who successfully established 70-80% of the rooted shoots of P. vera.

Direct rooting in vivo seems to offer better possibilities for establishing in vitro-propagated Pistacia material since 100% rooting was achieved at 500 mg l⁻¹ IBA. At 100 mg l⁻¹ IBA, 77.8% rooting was obtained. This result suggests that there is a possibility for improving the latter rooting levels by increasing the time of treatment above 5 s. Martinelli (1988) obtained the best rooting of P. integerrima seedling material in vivo following a "quick" dip in 100 mg l⁻¹ IBA. However, the precise duration of dipping was not mentioned.

Comparing the rooting responses of seedling material obtained in the above experiment with those of microshoots derived from four-year-old P. vera material (Section 4.3.2) shows that the former type of material responded to lower IBA and time of treatment than the latter one thus confirming, once again, the difficulties of propagating mature Pistacia material.

These results indicated the ease with which P. vera seedling material can be acclimatized in compost mixtures in the greenhouse under mist conditions, either by transferring in vitro rooted shoots to these in vivo conditions, or better still, by direct rooting of these micro-shoots in vivo.

CHAPTER FIVE

MICROGRAFTING OF PISTACHIO

CHAPTER FIVE : MICROGRAFTING OF PISTACHIO

5.1 IN VITRO MICROGRAFTING

Because of difficulties in rooting cuttings of pistachio cultivars (Assaf, 1971, Joley and Opitz, 1971; Al Barazi and Schwabe, 1982), grafting and budding onto seedling rootstocks is the main alternative being practiced to vegetatively propagate this species (Woodroof, 1979, Hartmann and Kester, 1983).

Micrografting, which is an accommodation of grafting procedures to tissue culture is performed either in vitro or in vivo and is increasingly being used in fruit and nut tree species for different purposes. These include the production of disease-free scions (Murashige et al., 1972; Navarro et al., 1975), the rejuvenation and/or invigoration of mature shoot materials (Franclet, 1979; Hackett, 1985) and the study of the graft unions (Martinez, 1979⁽¹⁾; Gebhardt and Goldbach, 1988). Pistachio has benefitted little from the use of this relatively new approach. The only available information on micrografting in this nut species is concerning the first attempts to rejuvenate mature material by micrografting in vitro mature scion meristem-tips onto juvenile P. vera rootstocks (Barghchi and Martinelli, 1984; Barghchi, 1985a). However, a very slow growth of the grafted scion was observed and no elongation was ever obtained in these studies despite supplementation of GA₃ to the culture medium (Barghchi, 1985a). Neither were histological studies carried out in the above work in order to investigate connections between scions and rootstocks. An urgent need for developing a working micrografting system for pistachio was therefore evident since this approach would benefit this important and recalcitrant species.

The aims of the present study were first to improve the system described by Barghchi (1985a) and second, to provide evidence if there is any of the establishment of functional graft unions. Besides investigating the in vitro approach, a method of micrografting pistachio in vivo was developed following a series of preliminary micrografting trials carried out on a much faster growing partnership, i.e., grafts between two solanaceous genera Nicotiana tabacum L. (NT)

(1) In Jonard (1986)

and Solanum aviculare Forst (SA) (a woody perennial).

5.1.1 In vitro micrografting of SA/NT

Besides providing a fast growing system for investigations, the SA/NT combination is useful as a model system to study the processes of rejuvenation and/or invigoration as influenced by grafting. Moreover, this system provides a suitable combination for studying not only chimera production, but also, the formation of the graft union because of the compatibility of the two different types of tissues which are easily identifiable. This system is currently being used in vivo to investigate the first and second above-cited subjects (Kaddoura, 1989). In the following experiments, the results of the first attempts to micrograft SA and NT in vitro are presented. The effects of the combination used (NT/SA vs SA/NT) and of the type of rootstock (germinating seedling vs micropropagated shoots) were investigated.

Seeds of SA and NT were surface sterilized in 10% 'Chlorox' for 20 min and germinated in petri dishes on agar (7 gl^{-1})-solidified MS medium. Cultures were incubated at 25°C in darkness. Two weeks later, developing seedlings were either subcultured on MS medium containing 20 gl^{-1} sucrose and solidified with 7 gl^{-1} agar for elongation or on an identical medium but supplemented with 2 mg l^{-1} BAP and 0.2 mg l^{-1} NAA for proliferation. Cultures were incubated at $25^{\circ}\text{C}/8\text{h}$ photoperiod. In the case of micropropagated shoots, rooting was performed on hormone-free $1/2 \times$ MS medium at $25^{\circ}\text{C}/8\text{h}$. The two micrograft combinations scion/stock were NT/SA and SA/NT. Scions which consisted of apical meristems and the adjacent tissues including 3 to 5 leaf primordia were excised under a binocular microscope ($\times 10-20$). Rootstocks were obtained either directly from germinated seeds or from micropropagated shoots. They were decapitated leaving 10-15 mm of the epicotyl, the cotyledons removed and the root system shortened to 20-25 mm. The scion was placed on top of the cut surface of the decapitated epicotyl in contact with the vascular ring. Micrografts were maintained upright on agar(7 gl^{-1})-solidified MS medium and were incubated at $25^{\circ}\text{C}/8\text{h}$ for four weeks before they were transferred to soil. Ten micrografts were performed for each type of rootstock and for both scion combinations. Successful micrografts were potted in a

mixture of peat and vermiculite (1:1, V:V) in April-May, and were kept in covered propagator trays in a fogging unit ($95 \pm 5\%$ RH) and the vents in the lids gradually opened over the subsequent three-week period when the micrografted plants were transferred, uncovered, to a second fogging unit held at $65 \pm 5\%$ RH for a further three to four weeks before they were moved into normal glasshouse conditions.

The first attempts to micrograft SA and NT in vitro were successful (90-100% success levels); 100% being achieved when in vitro rooted shoots were used and 90% obtained when germinating seedlings were used as rootstocks. This was achieved in the case of both the combinations NT/SA and SA/NT (Plates 11 and 12). Similarly, weaning was very easy and 100% of successful micrografts were established in vivo independently from the types of combination or rootstock (Plates 13 and 14).

5.1.2 In vitro micrografting of pistachio

The only information available from the report of Barghchi (1985a) is that juvenile P. vera rootstocks and meristem-tips containing a few leaf primordia were used; the micrografting procedure consisting of placing the scion explants on top of the cut surfaces of decapitated seedlings or inside a vertical cut made on the side of the rootstocks. No details of the age and preparation of the rootstocks, the level of the grafts, the type of media used (liquid or solidified) nor of the conditions under which the micrografts were maintained were reported.

The method described by Murashige et al. (1972) and Navarro et al. (1975) for Citrus constitutes the basis of the approaches used so far and reported by most research workers. This consists of using two-week-old dark-grown seedlings as rootstocks and 0.14-0.18 mm long shoot-tips as scions, excised from flushes of field/glasshouse grown plants or from in vitro cultures. The cotyledons are eliminated and the epicotyls and roots are shortened to 1.5 cm and 4-6 cm, respectively. The scions are either placed on top of the cut surfaces of the decapitated epicotyls, or better, inserted into inverted-T incisions made at the points of decapitation of the epicotyls. Maintenance of the micrografts is performed in liquid medium using a

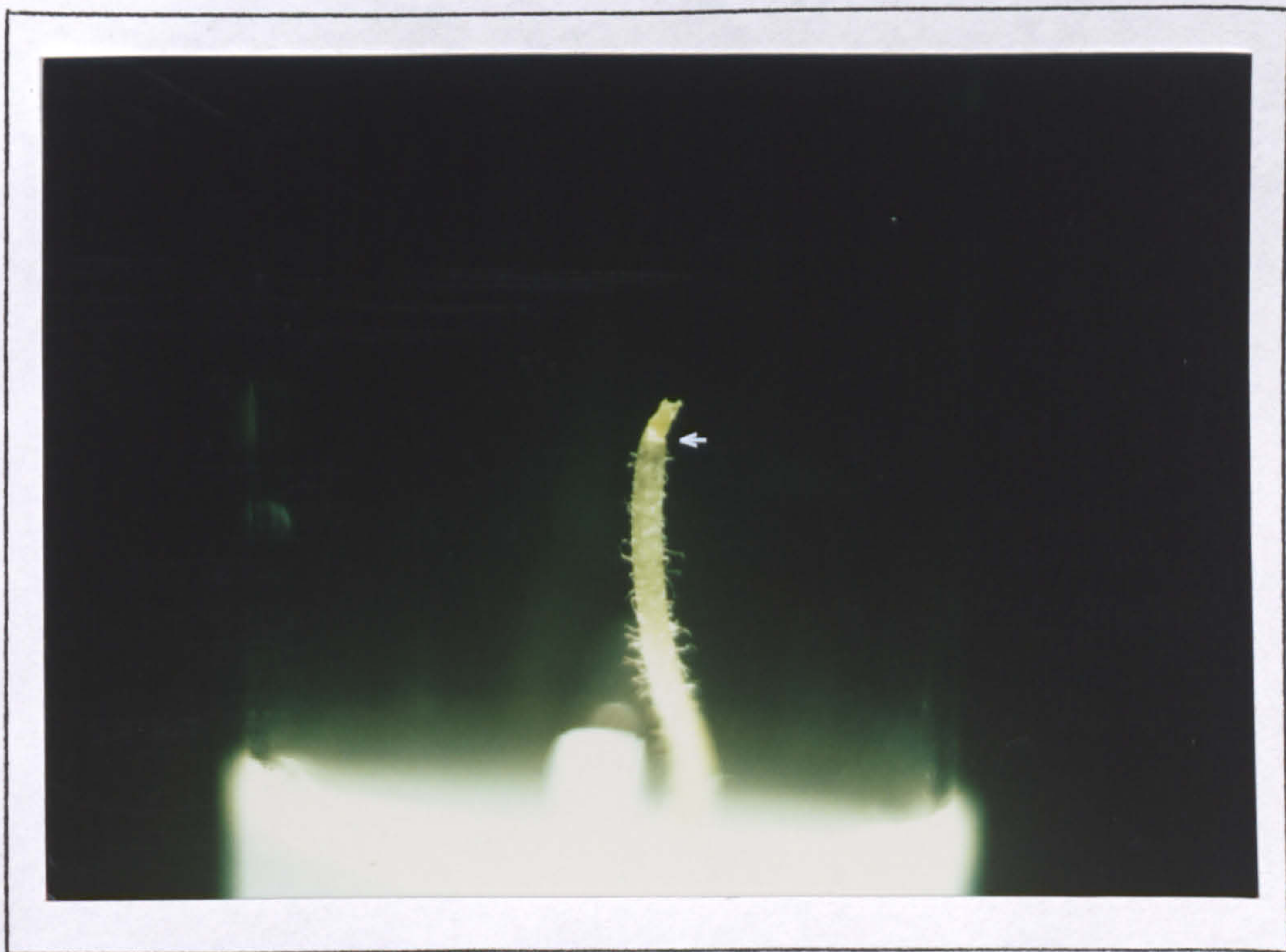


PLATE 11 : Meristem-tips of NT micrografted onto SA seedling rootstocks
in vitro after 2 weeks in culture.

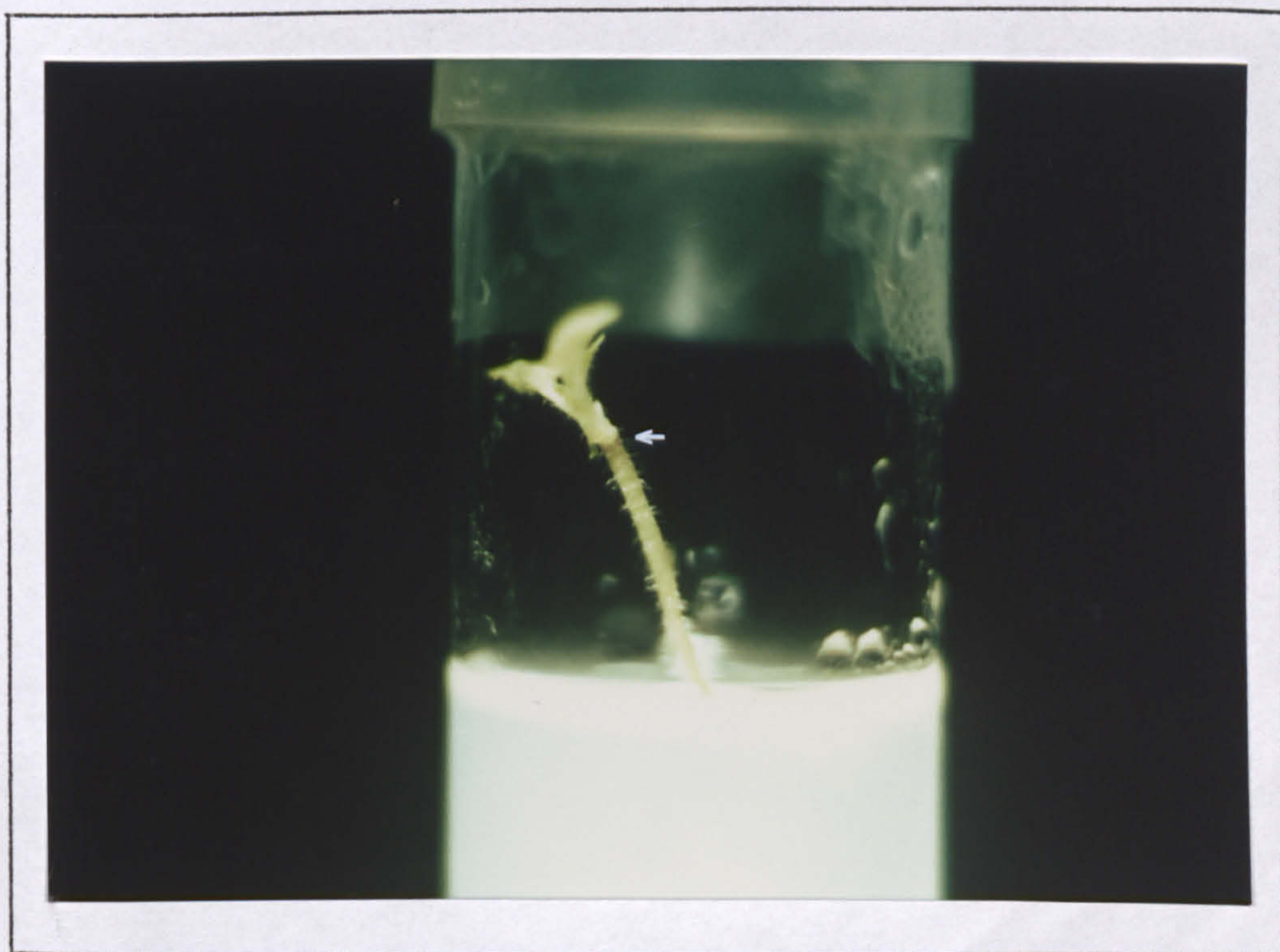


PLATE 12 : Meristem-tips of NT micrografted onto SA seedling rootstocks
in vitro after 3 weeks in culture.

PLATE 13 : Micrografts of NT/SA successfully transferred to glasshouse conditions after 2.5 weeks from potting.

PLATE 14 : Micrografts of NT/SA successfully transferred to glasshouse conditions after 13 weeks from potting.



filter paper bridge system for support and incubation is conducted under 16h daily exposure to 10,000 lux.

In the following experiments, the development of a model system for pistachio was considered and the effects of several factors on the graft success were investigated; these included the type and method of preparation of rootstocks, the sucrose levels added to the culture medium and the size of scions used.

5.1.2.1 First approach and preliminary results

a) Rootstock preparation

Rootstocks were grown from P. vera seeds in the standard way being individually cultured on top of filter paper bridges in tubes containing liquid $1/2 \times$ MS medium. Cultures were incubated at 25°C/16h photoperiod for 12 days before grafting work was carried out.

b) Scion preparation

The approach used consisted of using Stage II-derived shoots of four-year-old P. vera. This was adopted especially since the main objective of the current investigation was to develop a working system which could be used in rejuvenating and/or invigorating mature Pistacia material. The latter approach was essential, firstly, to avoid excessive browning and secondly, to provide better chances of success because of the relative vigour and faster growth rates of the younger scions.

Scions consisted of 10 mm long in vitro propagated shoots (subcultured $\times 4$) with 2 to 3 axillary buds. The basal end of the scion was cut into a gently sloping wedge over c. 3 mm long.

c) Grafting procedure

Twelve-day-old seedlings were decapitated leaving about 15-20 mm of the epicotyls, the roots were shortened to 30-40 mm and the cotyledons and their axillary buds removed. The scions were then inserted into vertical splits made in the centre of the cut surface of the

decapitated epicotyls with a fine sharp scalpel.

d) Maintenance of the micrografts

Micrografted seedlings were cultured on MS liquid medium containing 30 g l⁻¹ sucrose using filter paper bridges each perforated in the centre of its platform to hold the seedling in an upright position. Cultures were incubated at 25°C in darkness for one week before they were transferred to 16h photoperiod held at the same temperature.

After four weeks in culture, all the grafts had already formed white callus at the unions and 93.8% successfully established micrografts (Table 29). However, poor elongation of the scions was observed and no axillary shoots developed. At this stage, the decapitated roots did not branch; instead they became swollen, dark and necrotic. Unsuccessful micrografts browned and died.

5.1.2.2 Modifications of the procedure of preparation of rootstocks

a) Tap root left intact (grafting outside tubes)

In order to prevent swelling and necrosis of the root which might have contributed to the limited growth of scions, each tap root was left intact without being shortened and a similar experiment to the one cited above was conducted.

Being very long, the tap root bent and curled around the bottom of each of the culture vessels. This made handling of rootstocks very difficult once plantlets were removed from tubes. Moreover, returning rootstocks to the tubes after performing grafting was delicate and frequently the fragile roots broke. Consequently, this procedure was abandoned and alternative ones investigated.

b) Micrografting in situ (inside tubes)

In order to prevent damaging the root systems during handling of micrografts, carrying out grafting with the rootstock still inside the culture vessel was next attempted. An experiment was set up as

described above, except that the root systems were kept intact and the grafting was performed without moving the rootstocks outside the vessel. The medium used for germination was removed and replaced with 10 ml of liquid MS medium supplemented with 30 g l⁻¹ sucrose. Stage II-derived shoots (subcultured x 5) were used in this experiment.

Results presented in Table 29 showed a better growth of the scions with a mean elongation of 8 mm. Grafting success achieved was 90% and callus development was observed in all graft unions (Table 29, Plate 15). Although an improvement in scion growth was observed, performing the grafts in situ was delicate and time consuming and, therefore, further improvements were still needed.

c) The use of a 'Milcap' system (grafting outside tubes)

The next step after the failure of the previous approach was to provide a convenient working system for carrying out the micrografting itself. Attention was directed, therefore, towards finding another substitute to the filter paper bridge system. The main goals were to promote root development while at the same time ease both manipulations during micrografting and the transfer of micrografts to culture vessels and so reduce the amount of damage to tissues and the dislocation of the newly positioned scions. The system used by Deogratias et al. (1986a), for Prunus micrografting employed 'Milcap' which is a synthetic support made from polypropylene fibres and in combination with liquid medium, seemed to satisfy the objectives which had to be achieved and consequently it was considered appropriate for investigation.

Following the adoption of the 'Milcap' support for the next investigations, a new strategy for germinating seeds was followed. This consisted of germinating P. vera seeds directly on top of the 'Milcap' supports instead of using filter paper bridges. After twelve days of culture, micrografting was performed as described for the filter paper system except that root systems were kept intact. In vitro propagated shoots (subcultured x 6) of four-year-old P. vera were used as scions.

TABLE 29 : Success rate and growth of four-year-old derived shoots of P. vera micrografts after four weeks of culture

Scion growth						
	Number of Micrografts	% Successful micrografts	% Callus (graft union)	% Axillary shoot development	Length increment ^a (mm)	Number of new buds ^a
<u>Micro grafting in vitro</u>						
Filter paper bridge system (tap root shortened)	16	93.8	100.0	0.0	3.2 ± 0.52	0.0 ± 0.0
'Milcap' system	20	100.0	100.0	60.0	12.5 ± 1.16	5.7 ± 0.59
<u>Micro grafting in situ</u>						
Filter paper bridge system (tap root left intact)	10	90.0	100.0	44.4	8.3 ± 0.74	3.0 ± 0.29

^a Mean ± SE.



PLATE 15 : Four-year-old P. vera-derived shoot (Stage II) micrografted in situ onto a P. vera seedling rootstock after 5 weeks in culture.

The 'Milcap' supports proved excellent since they allowed good growth and branching of root systems. They resulted in limited damage to micrografts during transfers and generally facilitated all manipulations. Moreover, the growth of scions was noticeable (c. 12 mm length increment) and 60% of the scions developed axillary shoots. Callus was visible at all graft unions and 100% micrografts were successful (Table 29).

5.1.2.3 Effects of sucrose levels on micro grafting success

The sucrose content of the culture medium used to maintain micrografts has been reported to affect micrografting success in Citrus species (Navarro et al., 1975) but not in Prunus (Alskief and Villemur, 1978). Because of this controversy, and since no information on the response of pistachio micrografts to sucrose was available in the literature, the effects of this factor (at 30, 50 and 70 g l⁻¹) was investigated.

The results obtained (Table 30) showed that increasing sucrose levels in the culture medium above 30 g l⁻¹ was detrimental to micrografting success and to the development of scions. The main visible effect of high sucrose levels was on rootstocks; at least 50% of the stocks and 25% of the scions became necrotic and died. No such symptoms were ever observed on the shoots of the control treatment with 30 g l⁻¹ sucrose.

TABLE 30 : Effects of sucrose level in the culture medium on success of pistachio micrografts after four weeks of culture

	Sucrose (g l ⁻¹)			X ² _{test} (2 df)
	30	50	70	
Number of Micrografts	12	12	16	
% Success	100.0	75.0	62.5	(P>0.05)
% Necrotic rootstocks	0.0	50.0	56.3	(P<0.05)
% Axillary shoot development	58.3	41.7	25.0	(P>0.05)

5.1.2.4 Effects of scion size on micrografting success

The size of scions has been reported to influence the rejuvenation of mature materials; in general the smaller the scion the better the rejuvenation level achieved. This has been reported in several species such as Eucalyptus (Franclet, 1979). Based on this information, and in order to provide a better model system, an experiment was set up to investigate the smallest size which would be possible to use without reducing the level of success already obtained with larger scions (10 mm in length).

Scions were excised from Stage II-derived cultures (subcultured x 6) of four-year-old P. vera material. Micrografting was performed under a 'Zeiss' binocular microscope (x10-x40) provided with a cool light system. Scions of four classes of size were used: <0.3, 0.5-0.7, 1-2 and 2.5-3.0 mm long. They were excised from shoot-tip cultures and were placed on the cut surfaces of decapitated epicotyls in contact with the vascular ring. The 'Milcap' system and a MS liquid medium supplemented with 30 g l⁻¹ sucrose were used to maintain the micrografts in vitro. Cultures were incubated in darkness for one week before they were transferred into light (16h) conditions.

The success levels obtained increased with the size of the scions used (Table 31). All the meristem-tips <0.3 mm became necrotic and only a third of the scions measuring 0.5-0.7 mm survived. The best results were obtained with scions of 2.5-3.0 mm long meristems which gave 91.7% successful grafts.

TABLE 31 : Effects of scion size on success of pistachio micrografts after four weeks of culture (n=12)

	Scion size (mm)				x ² _{test} (3 df)
	<0.3	0.5-0.7	1.0-2.0	2.5-3.0	
% Successful micrografts	0.0	33.3	83.3	91.7	(P<0.05)

5.1.2.5 Micrografting onto in vitro rooted shoots

Another alternative to using seedlings as rootstocks would be the use of clonal in vitro rooted shoots. Such types of method have been used in the case of several fruit tree species such as in Prunus (Cornaggia, 1986). In the following experiment, the effects of using in vitro rooted shoots instead of seedlings as rootstocks on graft success and scion growth were investigated.

Rootstocks consisted of Stage II - derived cultures (Subcultured x1) of P. vera seedlings rooted in vitro according to the method described in Section 4.3.1.1a, and the scions obtained from Stage II cultures of four-year-old P. vera (subcultured x 2). Rootstocks of c. 20 mm long were induced to root on $1/2$ x MS medium containing 2 mg l^{-1} IBA and micrografted as described in Section 5.1.2.1, at the stage of growth when roots started to appear. Micrografts were maintained on agar (7 g l^{-1})-solidified full strength MS medium and were incubated for the first week in the dark before they were transferred to light (16h) conditions.

Grafting onto in vitro rooted shoots did not present any manipulation problems. Grafting success achieved was 91.7% and the mean elongation of scions recorded was 6.8 mm over four weeks. No axillary shoots developed on the scions. Callus was observed at all graft unions (Table 32).

5.2 In vivo Micrografting

Micrografting has also been performed in vivo as opposed to in vitro, mainly to rejuvenate mature plant material such as in forest trees (Franclet, 1967 in Jonard, 1986) and to produce virus-free plants such as in Prunus (Colin and Verhoyen, 1976) and in Citrus (Tusa et al., 1987). The main advantage of the method is to bypass the delicate stages of in vitro culture. Having developed a model system in vitro, in vivo micrografting of pistachio was attempted as an alternative to the in vitro approach, especially since the procedure, if successful, would not only reduce the expenses but also would provide an alternative to be used in situations where tissue culture facilities might be lacking.

TABLE 32 : Success rate and growth of four-year-old P. vera micrografts using
in vitro rooted shoots as rootstocks (n=12)

Scion growth				
% Successful micrografts	% Callus formation	% Axillary shoot development (mm)	Length increment ^a (mm)	No of new buds ^a
91.7	100.0	0.0	6.8 ± 0.84	1.1 ± 0.41

^a Mean ± SE (over four weeks).

Scions were obtained either from four-year-old P. vera-derived shoots (subcultured x 6 at Stage II) or, from shoots of seven-month-old glasshouse-grown P. vera seedlings. Rootstocks consisted of seven-week- or seven-month-old P. vera seedlings grown under glasshouse conditions in a mixture of peat and grit (1:1, v:v). Scion preparation and grafting procedures used were similar to those described above for in vitro micrografting (see Section 5.1.2.1) except that scions were c. 15-20 mm in length and grafting was performed at c. 70 and 130 mm ^{above} the substrate level in the case of the former and the latter types of rootstocks, respectively. Grafting was performed in September 1989 at Wye, and micrografts (12 replicates each) maintained as described in Section 3.4.

Successful micrografts were established in vivo in both of the combinations tested. After seven weeks of grafting, the success level was 83.3% in the case of scions micrografted onto seven-week-old seedlings. A slightly higher percentage of success (91.7%) was obtained in the case of homografts of seven-month-old seedling materials (Plate 16).

5.3 Histological studies on micrograft unions

Following the apparent successful micrografting of pistachio, histological studies were necessary to establish conclusively whether the micrografts formed complete graft partnerships. No such studies have yet been reported in the literature for pistachio.

In the following investigations, the formation of the graft union was followed over time using the most successful system developed in vitro (i.e., 'Milcap' system, Section 5.1.2.2c).

Four-year-old P. vera-derived shoots, obtained from Stage II cultures (subcultured x 6) were micrografted in vitro onto P. vera seedlings using the 'Milcap' system. Each of three graft unions were investigated at 3, 7 and 14 days after micrografting (using FM, Section 3.6) and at 21 days (using LM and TEM, Sections 3.5 and 3.7.2, respectively).



PLATE 16 : Successful in vivo micrografts of pistachio after seven weeks from grafting. (Left) : four-year-old P. vera-derived shoot (Stage II) micrografted onto seven-week-old P. vera seedling. (Right) : Homograft of seven-month-old in vivo micrografts of pistachio after seven weeks from grafting.

The observed sequence of events in the formation of the graft unions was as follows:

Day 3: Large numbers of fluorescing nuclei were observed indicating a high meristematic activity in tissues of both the scion and stock (Plate 17).

Day 7: A well established callus was formed along the graft interface thus forming a bridge between the scion and the stock. Both graft partners appeared to take part in the development of the formed callus bridge (Plate 18).

Day 14: Differentiating vascular tissues were observed within the newly formed tissues at the graft interface, on both sides of the callus bridge; these proliferating inwards towards each other. Xylem elements were identifiable at this stage (Plate 19).

Day 21: Bridging of the graft by vascular tissues was completed thus forming a continuous vascular connection system between the scion and the stock (Plate 20). At this stage, portions of the necrotic layer could still be seen along the graft separating the tissues of each partner. Electron microscopy investigations (TEM) showed that the remaining portions of the necrotic layer between the two tissues of the graft consisted of dead, crushed cells (Plate 21).

Light and fluorescent microscopy investigations showed, therefore, that cell division occurred as early as three days after micrografting and that vascular continuity was established across micrografts by three weeks after grafting.

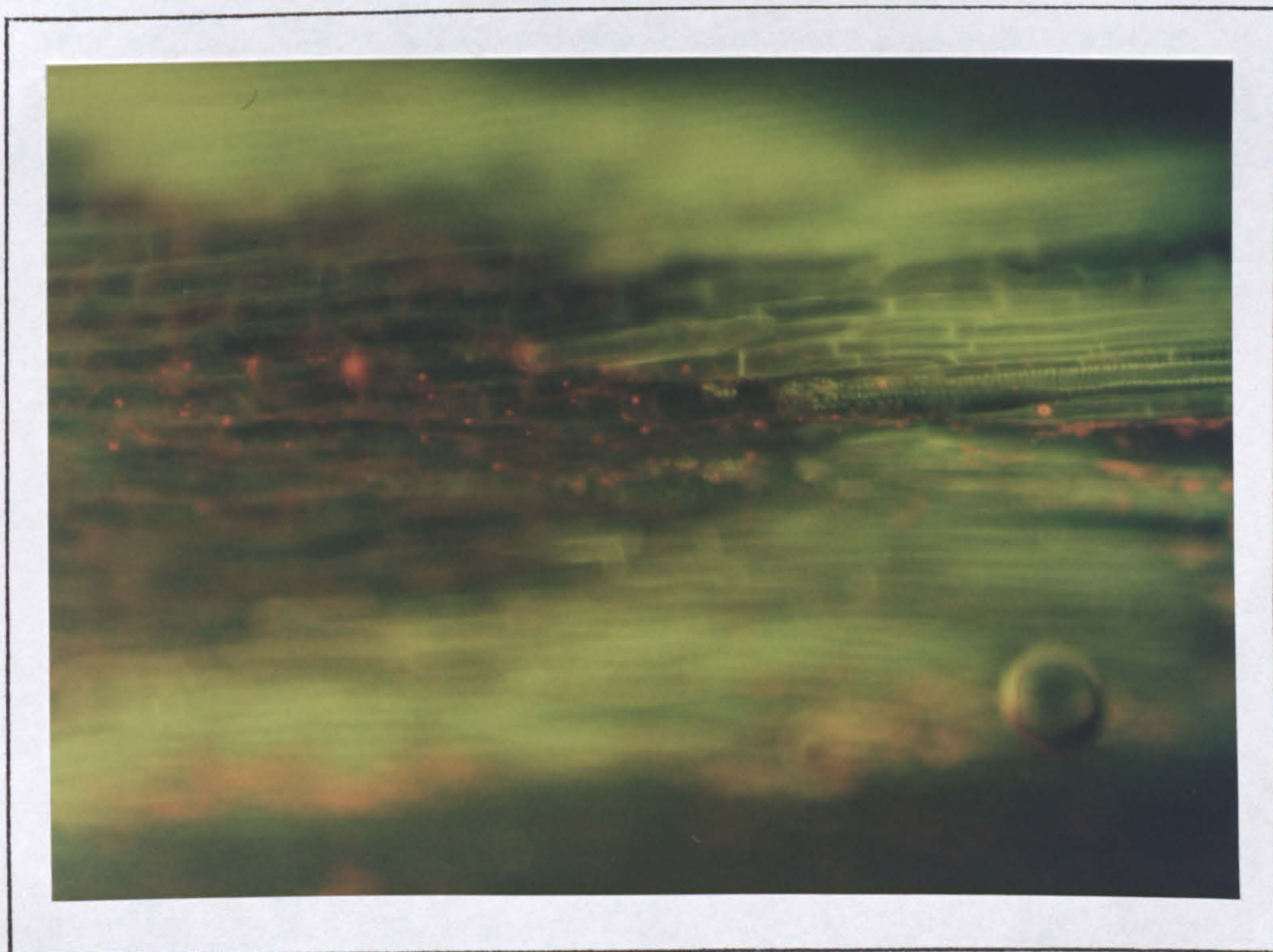


PLATE 17 : Fluorescing nuclei indicating a high meristematic activity at the micrograft union three days after micrografting in vitro of a four-year-old P. vera-derived shoot (Stage II) onto a P. vera seedling rootstock. (LG section, FM, X 150).

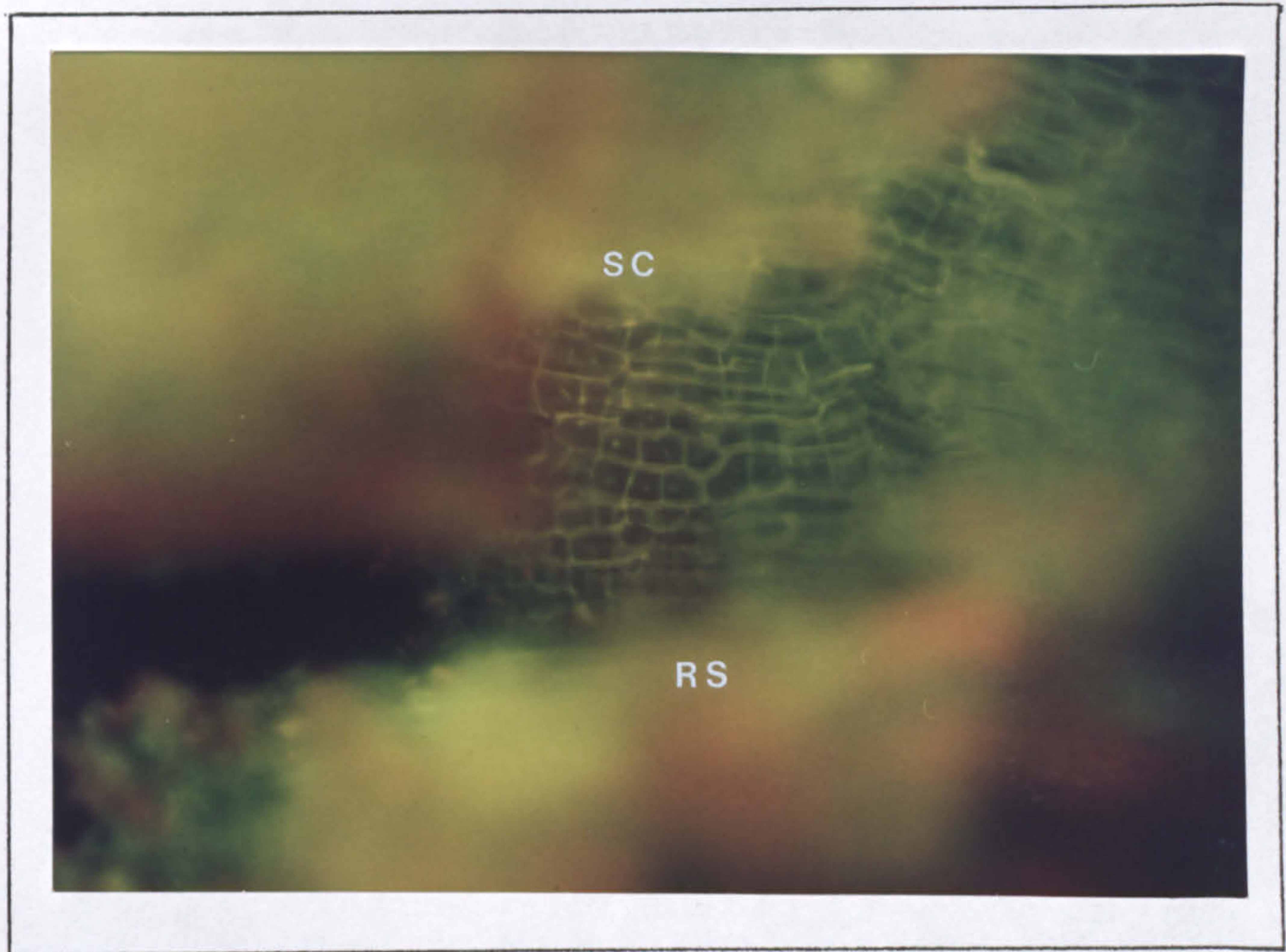


PLATE 18 : Callus bridge formation between the scion (SC) and the stock (RS) seven days following micrografting in vitro (LG section, FM, X 100).

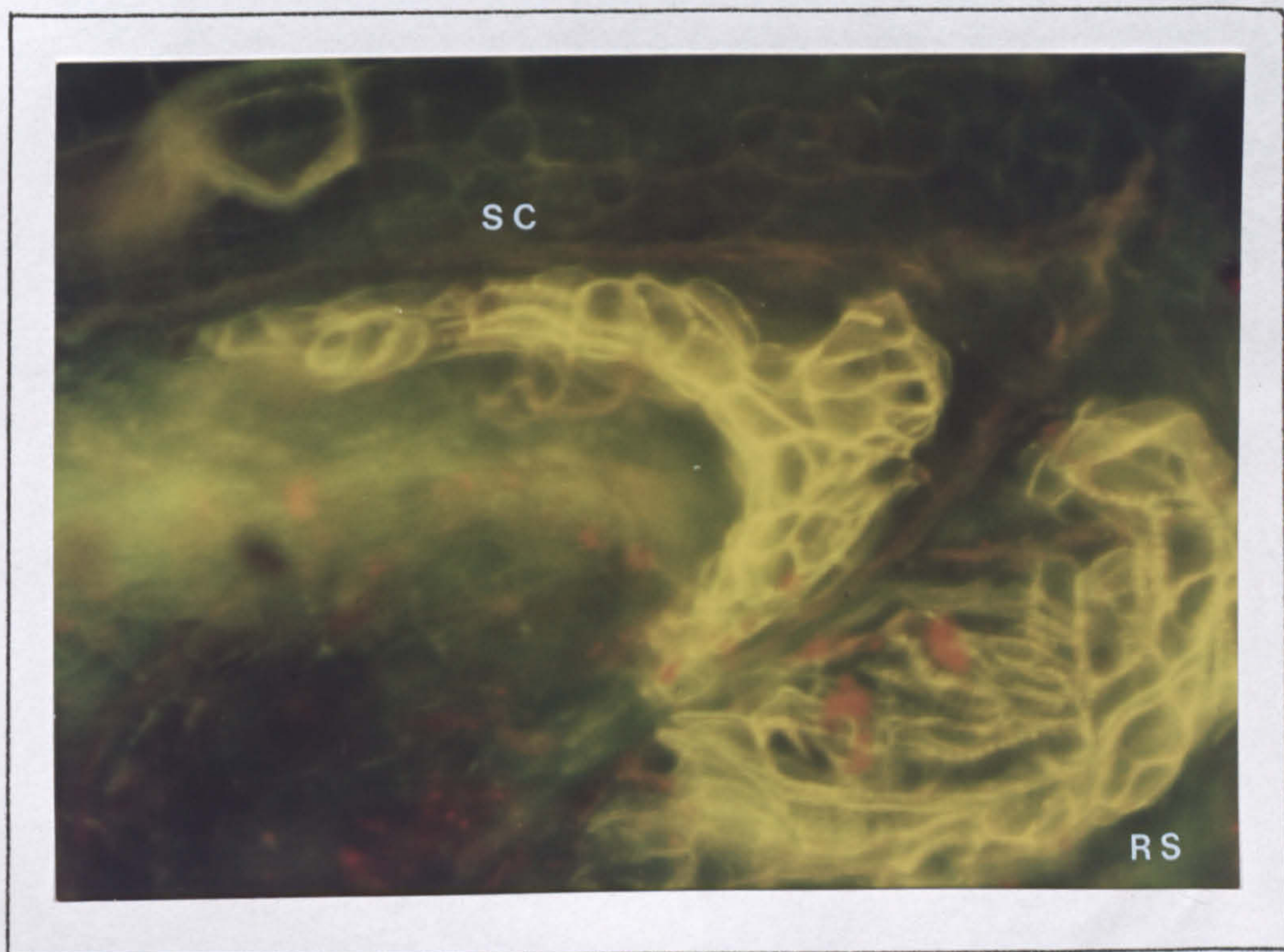


PLATE 19 : Fluorescing vascular tissues newly formed on both sides of the callus bridge and proliferating inwards after 14 days from micrografting in vitro scion (SC) and rootstock (RS). (LG section, FM, X 200).

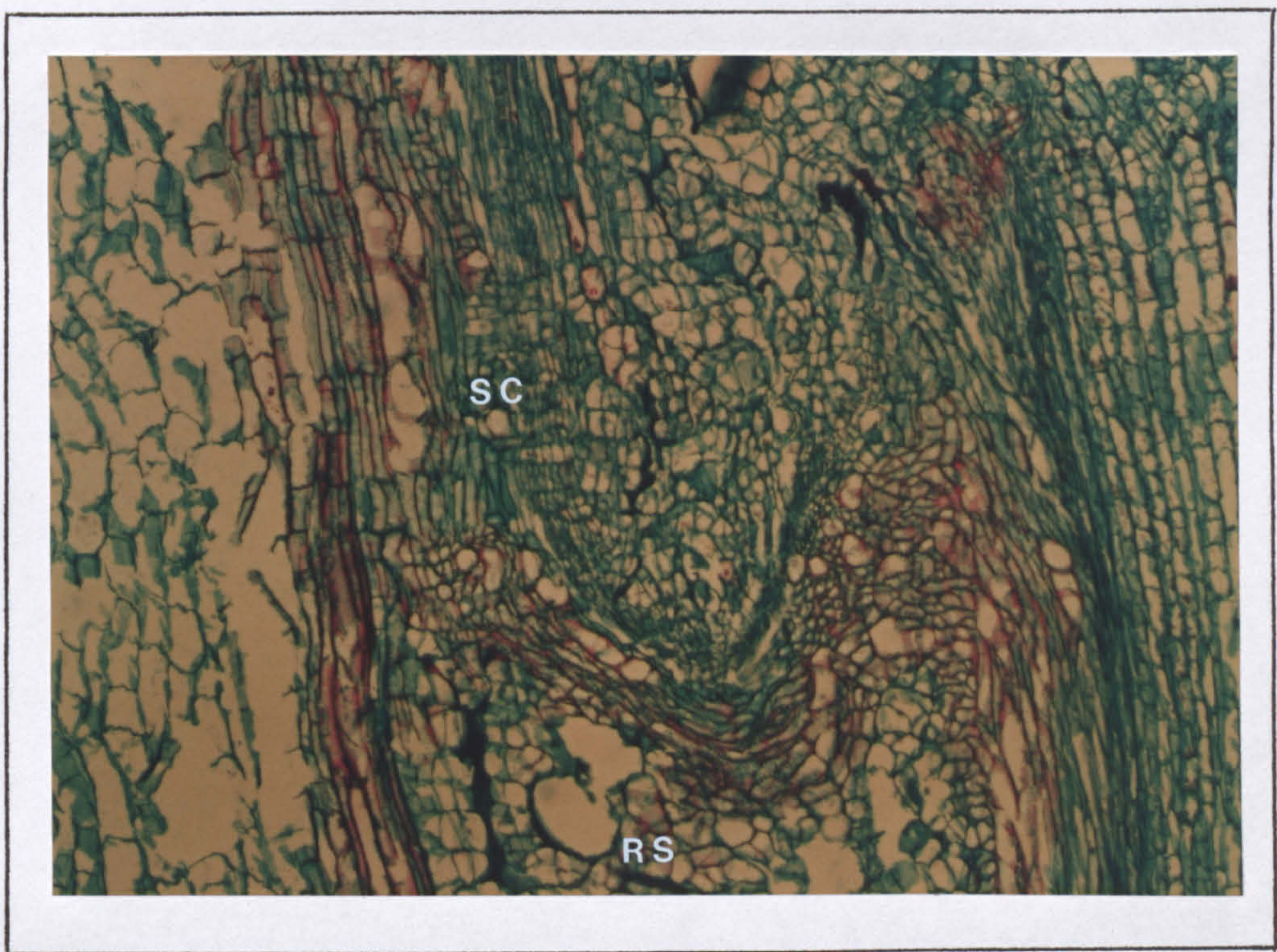


PLATE 20 : Bridging of the graft by vascular tissues and a continuous vascular connection established between scion (SC) and rootstock (RS) after 21 days from micrografting in vitro. (LG section, LM, X 100).

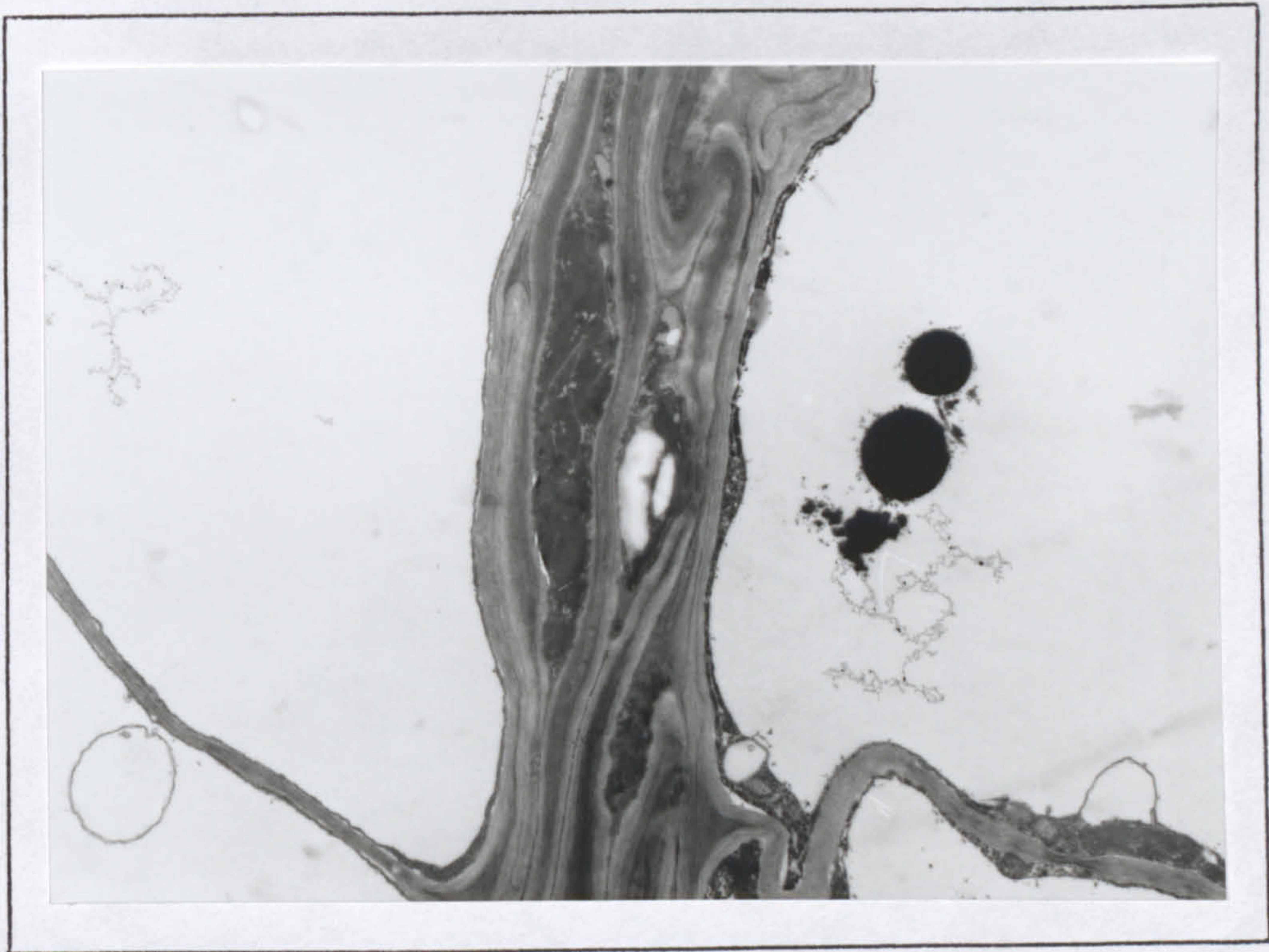


PLATE 21 : Electron micrograph showing dead crushed cells at the remaining portions of the necrotic layer observed at the graft interface after three weeks from micrografting in vitro. (LG section, TEM, X 7,500).

5.4 Discussion

Developing a workable micrografting model system for pistachio is of great importance if this species is to benefit from the tremendous advantages of the micrografting techniques and from which other species such as Citrus (Navarro, 1984), Prunus (Deogratias et al., 1986) and Eucalyptus (Franclet, 1979) have already benefited greatly. The main objective, for the time being, would seem to be an application of the above technique to produce rejuvenated elite materials of Pistacia mature shoots which have been reported to be recalcitrant as far as their vegetative manipulation is concerned (Joley and Opitz, 1971; Al Barazi and Schwabe, 1982; Martinelli, 1988). This fact has been confirmed also by the results of the current studies on in vitro rooting (Section 4.3.2).

In order to avoid any misinterpretation of the obtained results on pistachio (due to improper manipulation) the first trials were with SA and NT, a fast growing partnership (Section 5.1.1). Using this system, 100% successful micrografts were obtained in vitro following the micrografting of meristem-tips (the apical meristem + 3-5 leaf primordia) of either SA or NT onto in vitro propagated shoots of NT and SA, respectively. A slightly reduced level of take (90%) was obtained when germinating seedlings were used as rootstocks. This seemed to be related to the thickness of the rootstocks used; the latter types having much smaller decapitated surfaces which made the operation of micrografting even more intricate. Weaning of micrografts was also successful and no problems were encountered with the establishment of micrografted plants.

Having achieved these results on a model system, it ensured that the technique being used for micrografting was most likely to be sufficiently adequate to then be applied to pistachio. Moreover, the system developed in vitro as opposed to the one which is commonly used in vivo would provide a model system to study (under controlled environments) firstly, the processes of rejuvenation and/or invigoration as influenced by grafting mature SA onto NT and vice versa; secondly, the processes leading to successful graft formation since two different but compatible genera are involved so that the two

individual tissues can readily be identified following routine histological analysis and, thirdly, the establishment of chimeras artificially by micrografting the two compatible species together. The first and the third approaches have already been the subject of intensive investigation (Kaddoura and Mantell, 1990).

Concerning pistachio, a poor response was observed when the method widely used for Citrus (Murashige et al., 1972; Navarro et al., 1975) was attempted within this genus. Although callus formed at all graft unions and 93.8% successful micrografts were obtained, a poor elongation of scions and a lack of branching of both scions and root systems characterized the obtained micrografts. In the latter case, the rootstock became swollen and necrotic (Section 5.1.2.1). The average time taken to complete each graft (including capping of tubes, etc.) was 4 min. Leaving the tap root intact was not any better than shortening it, since being fragile, it frequently broke. Moreover, this approach made manipulation delicate because the tap root elongated and curled around the bottom of the culture vessels (Section 5.1.2.2a). The time taken for each graft was in the order of 6 min. Micrografting in situ provided better growth of the scions but performing the grafts was a delicate operation and time consuming (Section 5.1.2.2b). Each graft took approximately 10 min to complete. The most reliable technique developed in this work consisted of using the 'Milcap' support (Section 5.1.2.2c). This approach permitted good growth and branching of the root system in addition to an absolute success of take of all pistachio micrografts carried out in this study. The method also allowed a better elongation of scions which also developed axillary shoots. Moreover, this system proved excellent in easing manipulation and prevention of root damage during micrografting operations. The mean time to complete each graft by this method was 2 min. The use of liquid medium combined with a filter paper-bridge system to maintain the grafts seemed to have resulted in asphyxiation of roots thus leading to their necrosis. In addition to protecting the root system from being damaged during manipulation, substituting the above support (i.e., filter paper bridge) with the 'Milcap' one may have improved oxygenation of roots and hence resulted in their better growth and branching. This effect in turn may have been one reason why scions were able to elongate more dramatically

following this treatment rather than any of the conventionally used ones. The results obtained thus confirmed the finding of Deogratias et al., (1986a) who came to a similar conclusion while working with Prunus. These findings also agreed with the observations made in the field which suggest that pistachio roots are sensitive to excess water in soil (Joley, 1979).

Supplementation of the culture media used to maintain the micrografts with 30 g l^{-1} sucrose proved optimal for grafting success and for subsequent scion growth. Increasing sucrose above this level resulted in a poor response (Section 5.1.2.3). These results are in agreement with those found on apple (Alskief and Villemur, 1978) in which 30 g l^{-1} was optimal and no successful grafts were obtained either with 60 g l^{-1} or with 75 g l^{-1} sucrose. However, in Citrus, Navarro et al. (1975) have reported an increase in the number of successful grafts employing increasing sucrose levels of up to 75 g l^{-1} . Above this level there is usually a reduction in grafting success. These differences might reflect differences in carbohydrate requirements of the various species mentioned above.

Since a workable micrografting system for pistachio would provide workers with a model system for use in rejuvenation and/or invigoration of mature pistachio shoot materials in order to achieve easier rooting of clonal materials, the size of the scion used (i.e., 10 mm long) would not be so crucial as it would be in the case of achieving virus elimination by micrografting. However, it is reported that a better rejuvenation level could be achieved with small scions (Franclet, 1979). The results presented in Section 5.1.2.4 showed that pistachio scions of 1-3 mm could be used to study rejuvenation of mature shoot material with success levels of 83-92%; slightly lower levels than those initially obtained with scions of 10 mm long. No successful micrografts were obtained for pistachio in the current work using scions <0.3 mm and this failure was turned into more successful levels (i.e., 33%) when shoot tips of 0.5-0.7 mm were used. The relationship between grafting success and scion size has also been reported in several species (Navarro et al., 1975; Huang and Millican, 1980). In Prunus for instance, no successful grafts were obtained with scions <0.4 mm in length. Attention should be drawn here to the fact

that in vitro propagated shoots were used as sources of shoot-tips in the current micrografting experiments. This approach has also been used by some workers and reported to have the advantage of overcoming the season-dependent responses of shoot-tips to micrografting (Navarro et al., 1975; Huang and Millican, 1980).

Micrografting shoot-tips onto in vitro rooted shoots was also attempted with success (Section 5.1.2.5). However, although callus formed at all graft unions, a slow growth and a lack of axillary shoot development on the scions was noticeable. This might partly be due to the fact that the scions used were subcultured only twice on multiplication medium (Stage II) as opposed to other cultures which had undergone 4 to 6 subcultures. In the latter cases, increased levels of scion elongation and branching were observed.

A system to successfully micrograft pistachio in vivo was also developed in the current work (Section 5.2). Interestingly, 83% success level was obtained when shoots derived from four-year-old P. vera (propagated in vitro, Stage II) were micrografted onto seven-week-old glasshouse-grown P. vera seedlings. Using a similar approach, Mosella et al., (1980) obtained 59 and 77% success by micrografting up to 12 mm long Prunus scions onto 30-40 and 80-120 day old seedling rootstocks, respectively. Being successful, such an approach would contribute greatly to reducing not only expenses, but also the need for reliance totally on in vitro approaches especially in situations where tissue culture facilities might be lacking.

Histological studies on micrograft unions showed that by 21 days a vascular connection was already established between the scions and the rootstocks (Section 5.3) thus providing evidence of the production of healthy and perfect graft unions. In a recent report on the formation of micrograft unions in Prunus, a complete xylem bridge was reported to be established after 27 days from micrografting (Gebhardt and Goldbach, 1988), i.e., six days later than in the case of the pistachio micrografting system developed in the current study. Active cell division was observed as early as three days following micrografting, and cellular events leading to the formation of pistachio micrograft union assembly resembled closely those events described by Hartmann and

Kester (1983) and McCully (1983) in typical graft situations. Three weeks after micrografting, portions of the disintegrating necrotic layer were still observed between tissues of the two partners. Investigation at the TEM level showed that these portions consisted of dead and crushed cells (Section 5.3). The necrotic layer is reported to consist of wounded and compacted cells at the cut surfaces of both partners (McCully, 1983). The compaction is probably caused by the expansion of the underlying cells especially in the place where scion and stock tissues have been in firm contact and where callus production has been restricted. The above worker also reported that the necrotic layer progressively disintegrates in successful grafts of entire dicotyledonous plants. Despite the fact that portions of a disintegrating necrotic layer persisted in sectioned graft (21 days following micrografting) it was quite clear from this investigation that bridging of the graft by vascular tissue was completed thus forming a continuous vascular system between both the partners.

In conclusion, micrografting of pistachio was successfully achieved in vitro as well as in vivo. In the former case, the use of the 'Milcap' support was crucial in providing a workable system by allowing a better root and shoot growth/development and by facilitating manipulations. The establishment of vascular connections between the stock and the scion provided further evidence that the model system being developed for pistachio was appropriate. Sucrose at 30 g l⁻¹ seemed convenient for maintaining micrografts and perfection of the technique was achieved by successfully micrografting scions of 1-3 mm long. Micrografting in vitro rooted shoots was also easily performed, thus providing a system which could be applied in case clonal rootstocks are to be used. Being successful, the in vivo micrografting approach provided other alternatives to the commonly adopted in vitro approach.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER SIX : GENERAL DISCUSSION AND CONCLUSIONS

The main objectives of the current study consisted of micropropagating and micrografting Pistacia materials used for commercial purposes in Morocco. Micropropagation of P. vera and P. atlantica rootstocks was investigated using seedling explants of aseptically germinated seeds. In the case of mature scion materials, explants were obtained either from four-year-old grafted plants, grown under glasshouse conditions in Morocco, or from 30-year-old field-grown trees of P. vera cv. Mateur. Concerning micrografting, the aim was to develop a workable model system for pistachio, which would undoubtedly benefit from the tremendous advantages that micrografting techniques have to offer as demonstrated by the significant successes that it has already enjoyed in the case of many fruit and nut tree species.

The results of the preliminary experiments on establishment of Pistacia materials (Stage IA) showed the ease with which seedling materials could be established as compared to shoots obtained from adult trees (i.e., four- and 30-years of age). The use of seeds previously stored dry at low temperatures was beneficial in avoiding excessive contamination of subsequent cultures using P. vera seeds. Storing Pistacia seeds, however, proved detrimental to seed germination as observed in P. atlantica seeds kept at 4°C for up to 12 months. In the latter species, scarification with sulphuric acid for 90 min was necessary prior to germination in vitro. The use of a vacuum to aid surface sterilization of Pistacia seeds was efficient in preventing fungal contamination. No pre-treatment of seeds with either temperature or growth regulators was needed to germinate seeds of both species and good germination and seedling growth was achieved on $1/2$ x MS liquid medium in the case of P. vera and full MS semi-solidified medium in the case of P. atlantica. Another approach to speed up and improve multiplication of seedling materials consisted of inducing multiple shoot formation from germinating seeds of P. vera and P. atlantica on media supplemented with 4 and 0.5-1.0mg l^{-1} BAP, respectively (Stage IB). Higher levels of BAP (6-8 mg l^{-1}) could still be used to obtain optimal response in the case of P. vera, but for treatment periods of much less than two weeks to prevent high levels of

vitrification associated with the presence of cytokinin in the media for a long duration.

In the case of adult pistachio materials, decontamination was best achieved with explants obtained from actively growing shoots of four-year-old P. vera grafted plants forced to develop under protected glasshouse conditions as opposed to shoots derived from 30-year-old field-grown P. vera trees. In the latter case, basal sections of summer-harvested shoots proved excellent sources of materials for establishment in vitro. Limiting manipulations and tissue injuries during the preparation and transfer of explants, surface sterilizing of explants with 20% bleach solution containing 0.1% Tween 20 for 20 min using a shaker and incubating cultures during the first stages of culture initiation in darkness for up to three weeks would seem to be satisfactory in limiting media browning and tissue injuries, and in producing good establishment results. No benefit was gained from the tested antibrowning compounds. This also applied to ascorbic acid despite the fact that this antioxidant proved efficient in preventing media browning in the leaf disc plating assay. Following the above procedures, up to 93% survival was achieved using four-year-old-derived explants. Although a high survival (85%) was obtained with explants derived from 30-year-old trees, maintaining cultures was difficult because of a slow decline in growth and increases in vitrification and chlorosis. Unfortunately, difficulties in securing permanent supplies of adult materials did not allow a continuation of investigations on the establishment of such mature materials.

In Stage II cultures, MS medium has proved superior for P. vera shoot multiplication when compared with the formulae of Anderson, WPM and Knop, (the latter medium being supplemented with MS micronutrients). However, this medium might still not be optimal because of the faint chlorosis it induced on leaves and the callus it stimulated at the base of shoots.

Multiplication of both seedling and four-year-old derived shoots of P. vera was optimal at 4 mg l^{-1} BAP. However, in the latter type of material which was already subcultured x 3, over half the cultures were vitrified when such^a relatively high levels of BAP was used. Much lower

vitrification levels developed at 3 mg l⁻¹ BAP, a level at which multiplication was not significantly different from that obtained at 4 mg l⁻¹. By contrast, in the case of P. atlantica seedling materials BAP at 1 mg l⁻¹ proved optimal. The continuous use of optimal levels of BAP produced dwarfed shoots with very small leaves and this type of material proved inappropriate for subsequent subculturing as well as for rooting. The use of low levels of 2iP in a mixture with suboptimal levels of BAP was beneficial in improving the growth and qualities of shoots without significantly affecting shoot multiplication at the tested levels. A mixture of 0.5 mg l⁻¹ 2iP and 1.5 mg l⁻¹ BAP proved optimal.

Growth and proliferation was also affected by the temperature at which cultures were incubated with significantly better performances being obtained at 25°C as opposed to 19°C. Combined temperature/photoperiod treatments also affected the responses of four-year-old P. vera-derived shoots. Improvement of shoot growth and proliferation, although not significant except for fresh weight, was observed at 29°C/12h as compared with 25°C/16h. It is not known if this response was due to the effects of temperature, photoperiod, VPD or to an interaction of all or some of these factors.

One of the most persistent problems during Stages I, II and III cultures of P. vera and P. atlantica was the development of STN symptoms. This disorder seemed to be associated with fast-growing conditions as shown in Stages IA and IB when increasing strengths of MS medium and optimal BAP levels were used, respectively, and in Stage II when optimal levels of BAP were used, when BAP was used as compared with 2iP, when cultures were incubated at 25 and 29°C as opposed to 19°C and when multiplication was performed on phytogel as compared with agar. The sequential development of visible symptoms and the results of the ultrastructure investigations suggested that STN is most likely to be a calcium- rather than a boron-related physiological disorder. Noticeably, increasing B level in the medium above 100 µM inhibited shoot growth and proliferation and adding 200 µM B even increased STN. In contrast, supplementation of culture media with Ca up to 30 mM, using Ca gluconate, clearly reduced STN but did not prevent its occurrence. This might be due to the high relative humidity and more

particularly to the low VPD within the culture vessels which could have depressed transpiration and resulted in an inefficient distribution of Ca in fast-growing shoot-tips. Actually, sealing of vessels (previously covered with 'Magenta' plastic lids) with nescofilm resulted in a rapid and prolific development of STN as compared with the behaviour of shoots in unsealed (control) vessels, and seedlings of rooted shoots grown under glasshouse conditions were symptomless as opposed to those grown in vitro. Moreover, STN symptom levels were more than doubled when cultures were grown in vitro at 25°C/16h than at 29°C/12h; VPD being c. 4 x higher under the latter than under the former environmental condition. A direct application of Ca to the most susceptible tissues (i.e., leaf- and meristem-tips) by a periodic inversion of tubes containing a liquid medium rich in Ca (15 mM) completely prevented the development of STN symptoms on P. vera seedlings under conditions where these would normally arise after only c. 12 days of cultures. Noticeably, shoot-tips of glasshouse grown P. vera seedlings had more than double the Ca contents of those obtained from shoots growing under in vitro conditions. The presence of phenolic compounds and the strong apical dominance characterizing pistachio might have contributed to the enhancement of STN symptoms.

Rooting was easily achieved in vitro with seedling materials of P. vera (75%) and P. atlantica (87.5%), with optimal responses achieved with explants subcultured once on multiplication media and induced to root on 2 and 1 mg l⁻¹ IBA, respectively. Rooting was improved in the case of P. vera seedling materials (83%) by keeping the levels of MS micronutrient at their full strengths. Explants taken from P. vera seedlings were also directly rooted in vivo following a "quick" dip (5 s) in concentrated IBA solutions; 100 and 77.7% rooting being obtained when 500 and 100 mg l⁻¹ IBA were used, respectively. As far as rooting of adult P. vera material is concerned, rooting was attempted in vitro using the quick dip method. Encouraging results were achieved with results showing, not surprisingly, lower responses of four-year-old P. vera derived shoots (previously maintained in vitro in Stage II) as compared with seedling material; optimal rooting (50%) being achieved when the cut ends of microcuttings were dipped for 10 s in 1000 mg l⁻¹ IBA. In vitro rooted shoots derived from P. vera seedling materials were successfully established in vitro with 81.8% survival being achieved.

Concerning the second part of this study, workable model systems to micrograft pistachio both in vitro and in vivo were successfully developed. In the case of in vitro method, use of the 'Milcap' system proved excellent not only by permitting a better growth and development of both root system and scion but also in facilitating the actual manipulations of micrografting. An absolute success in graft take was achieved following the micrografting of 10 mm-long scions of four-year-old P. vera-derived materials onto seedlings of P. vera grown in vitro. The technique was further perfected by micrografting scions 1-3 mm in length. In the latter cases, a range of 83.3 to 91.7% take was obtained. Maintaining micrografts on hormone-free MS liquid medium containing 30 g l⁻¹ sucrose was satisfactory. Micrografting in vitro onto rooted shoots obtained from P. vera seedlings was also promising and could offer possibilities of using in vitro rooted clonal material for micrografting studies. The successful take of these four-year-old P. vera-derived shoots may have been due to the fact that they had passed through a series of subcultures in vitro. This possibility needs testing in further work.

Another alternative to micrografting in vitro consisted of performing the micrografts directly in vivo. This was successfully achieved also and 83% take was obtained following micrografting in vitro propagated shoots of four-year-old P. vera derived materials onto seven-week-old glasshouse-grown P. vera seedlings.

Histological investigations of micrograft unions (performed in vitro) provided evidence that healthy graft unions were formed following the establishment of vascular connections between scions and rootstocks. Cell divisions at the graft interface occurred as early as three days following micrografting and, by three weeks, a vascular continuity was already established across grafts.

In order to generate more definitive information and to extend the results obtained in the current investigation, it is suggested that the following studies be made:

- a) In Stage I cultures, establishment of cropping pistachio materials needs great attention and it is to be investigated further. Among the more useful approaches will be the use of micrografting techniques to rejuvenate and/or invigorate mature plant materials. The micrografting techniques developed in the present work could be used in the future to study the effects of repeated micrografting on rejuvenation and/or invigoration in pistachio. However, before this can be done it will be necessary to obtain suitable markers at the biochemical level of juvenility and maturity in pistachio. This will involve the identification of specific proteins associated with either one of the two phases following PAGE and/or IEF separations of protein extracts. Using these markers it will then be possible to make more precise evaluations of the influences of any repeated grafting effects which could result in reversion of scions.

Considering the importance of phenolics in influencing the success of establishment with mature pistachio materials, emphasis should be placed on the time of the year explants are harvested and on the type, size and position of explants used. These factors need to be investigated further.

- b) In Stage II cultures, the main subject which needs to be studied further is that of STN. Most of the results obtained suggest that this is most likely to be a Ca-related disorder. The finding that "feeding" by direct application of Ca to shoot-tips prevented STN symptom development using seedlings needs to be applied to mature shoots on multiplication medium using double-phase media (i.e., liquid over solidified media).

Supplementation of Ca gluconate to the culture media together with cultural conditions providing high VPD seems to be a promising solution which needs further investigation.

More detailed studies involving the use of isotopes such as ^{45}Ca have recently been suggested by Barghchi and Alderson (1989) to quantitatively measure the availability of Ca during cell division and differentiation.

- c) In Stage III cultures, attention should be given to the development of direct rooting of micropropagated Pistacia shoots in vivo. Studies could consider using rejuvenated and/or invigorated materials obtained from micrografting. The durations and the concentrations of auxin treatments used with these different microprpagated materials will need close examination.

From the results of the current study, it is possible to put forward an outline scheme for the (micro)propagation and (micro)grafting of pistachio. This and estimation of numbers of rootstocks and scions which could be produced in vitro are presented in Tables 33 and 34, respectively.

In conclusion, it could be said that recent publications showed that tremendous effort is being devoted by different workers to propagate this recalcitrant species, pistachio. Further effort and collaboration are still needed to achieve this objective. I hope the above studies will contribute in one way or another to benefit this valuable crop thus serving not only the farmers in arid and semi-arid areas but also the environment itself through the probable benefits that pistachio cultivations could bring for the protection and development of such areas within Morocco.

TABLE 33 : Outline Scheme for (micro)propagation and (micro)grafting of pistachio

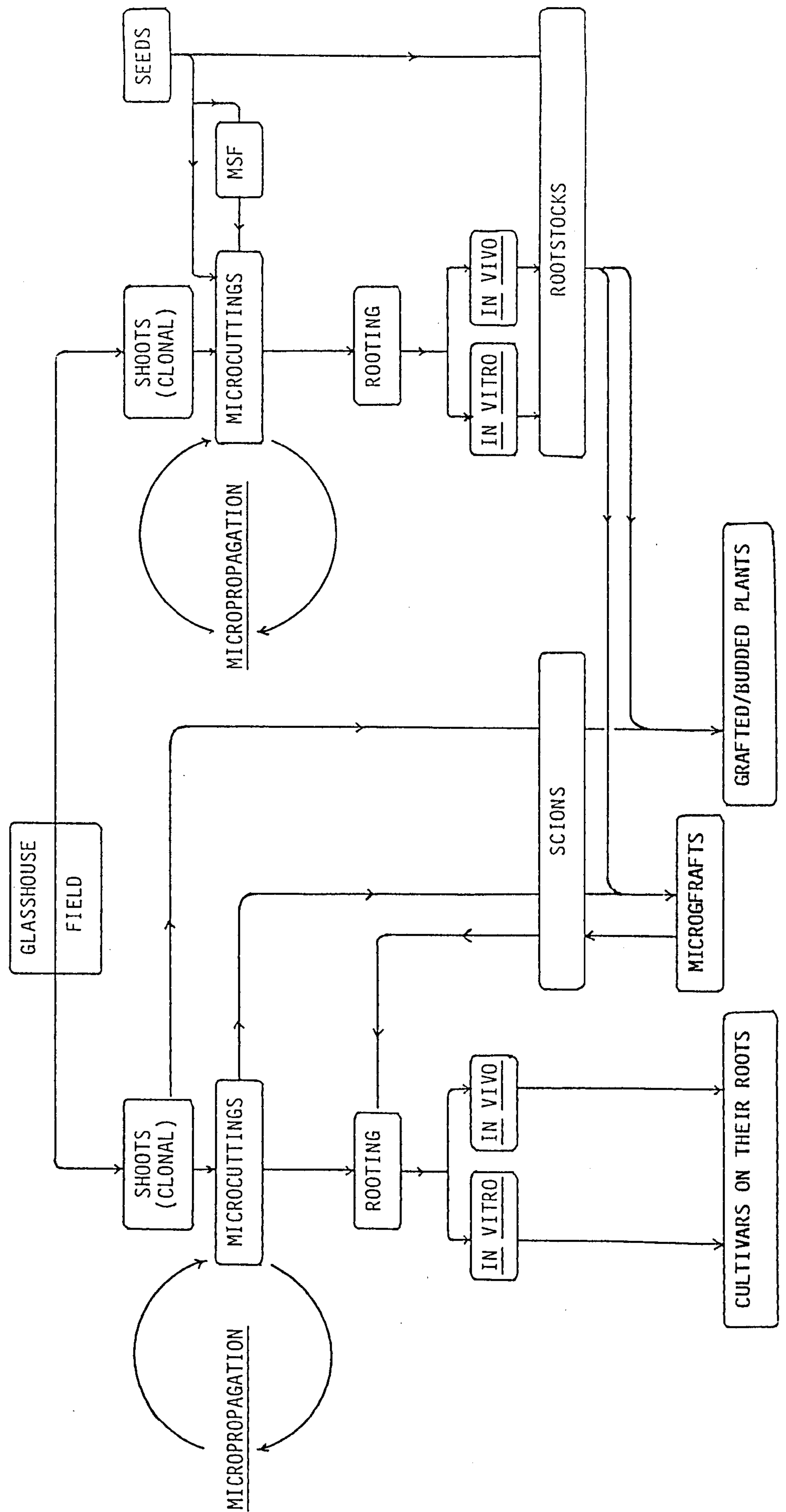


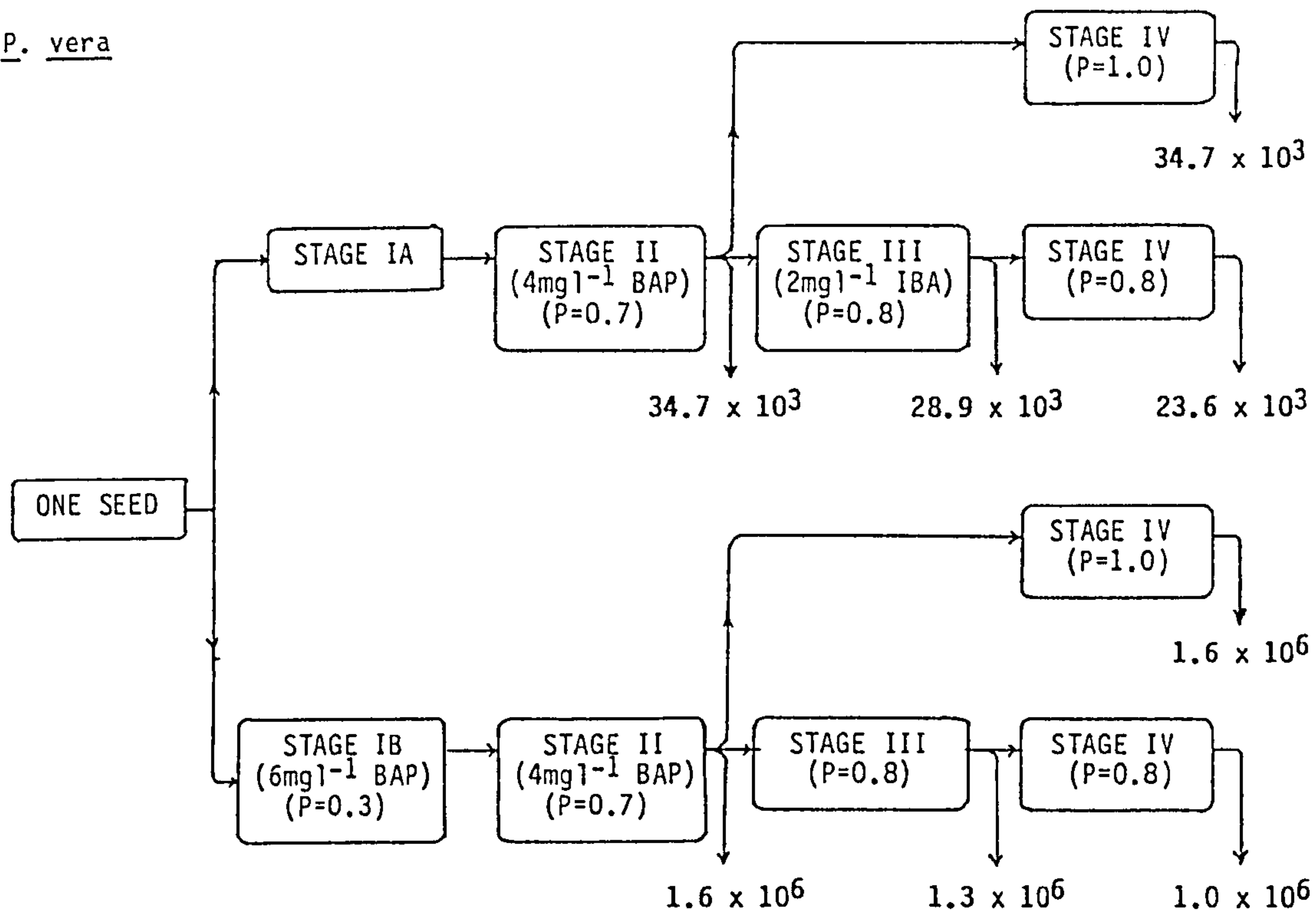
TABLE 34 : The approximate numbers of rootstocks and scions which could be produced from a single mother culture (seed for rootstocks and nodal explant of four-year-old plants for scions) over a period of 6 months (i.e., 6 generations of 4 weeks each, Stage II) were estimated using the following formula:

$$T = M^n \times C \times P$$

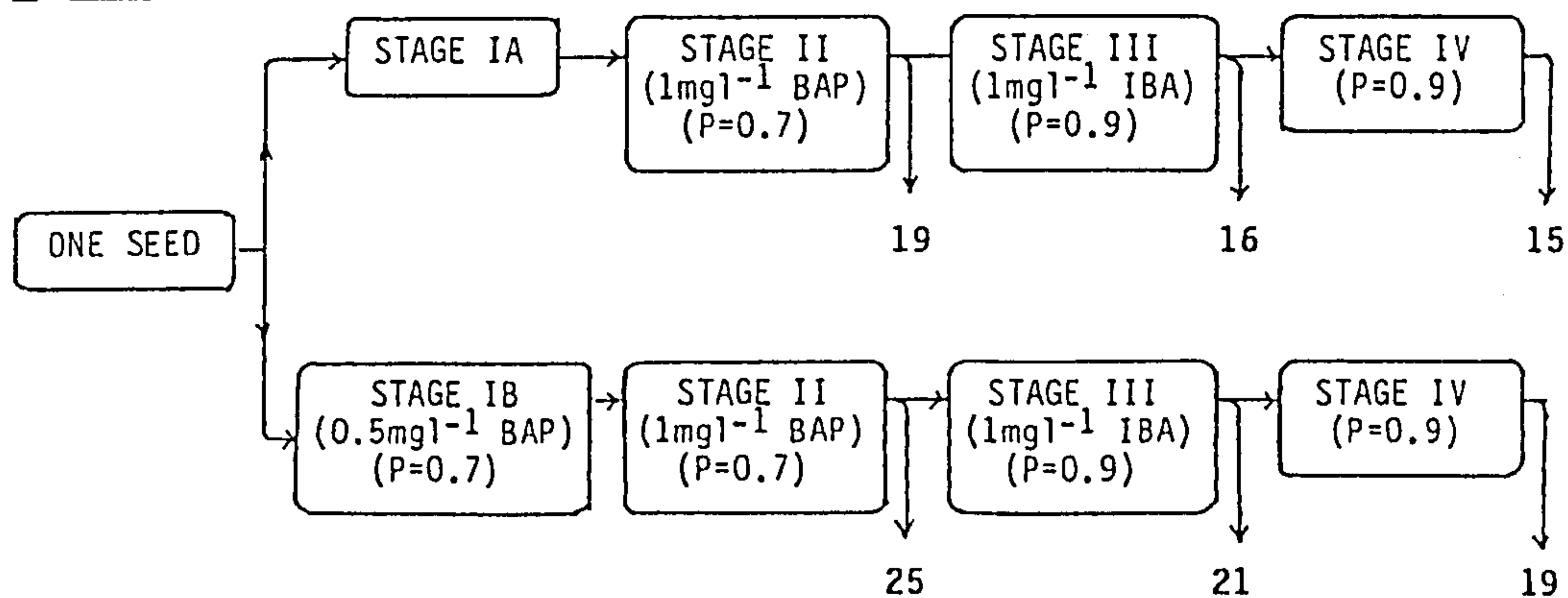
where T = total plants produced
M = multiplication rate
n = number of culture generations
C = number of starting cultures
P = proportion of culture materials remaining after vitrification, STN and infections.

1. PRODUCTION OF ROOTSTOCKS

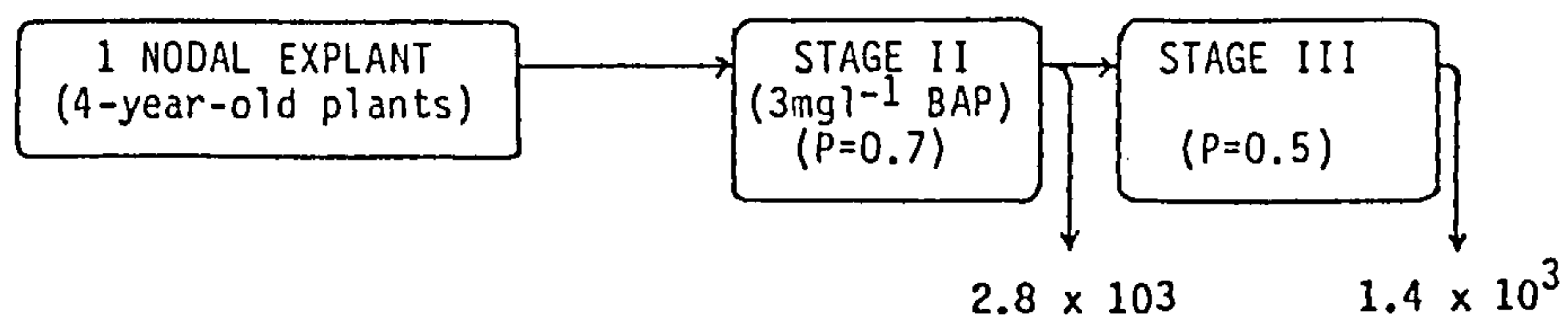
1.1 P. vera



1.2 P. atlantica



2. PRODUCTION OF SCIONS



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