OPTIMISATION OF PROPAGATION METHODS IN *Prunus persica* (L.) BATSCH.

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ABSTRACT

Propagating methods for peach (*Prunus persica* L. Batsch.) are currently limited to the use of seeds or cuttings. Most of the rootstocks commonly used for establishing peach trees commercially originate from a narrow genetic base. The most commonly used peach rootstock in South Africa is 'Kakamas', which has disadvantages such as slow growth, and a high susceptibility to certain pests and diseases. 'Kakamas' is classified as a mid to late ripening cultivar, hence, its use as a rootstock is restricted in early ripening cultivars. Optimising peach seedling and cutting production may on the other hand increase more selection material that will increase genetic variability and also serve as the basis for future production and selection of rootstocks for peach nursery establishments. Hence, this study was designed to improves success of peach cutting production by increasing the rooting percentage and the survival rate of cuttings. This was firstly achieved by the application of various concentrations of IBA and several rooting-cofactors. Secondly, rooting success of cuttings taken at different seasons was studied. The response of two cutting positions to various IBA and rooting-cofactor was evaluated. Attempts were also made to overcome the requirement of peaches for a seed stratification period by using plant growth regulators. Studies to optimise the establishment and growth of embryos of early to late ripening cultivars ('Klara', 'Oom Sarel' and 'Summer Giant') *in vitro* were undertaken to overcome the problem of small underdeveloped embryos which are often obtained from early ripening peach cultivars.

The early ripening peach cultivars 'DeWet', 'Earlibelle' and 'Florida Prince' were propagated by softwood, semi-hardwood and hardwood cuttings prepared from August 1999 to July 2000. It was found that softwood cuttings prepared in October and November responded well to an IBA application of 1000 mg l^{-1}, resulting in 100 % rooting in all three cultivars. In 'Florida Prince' and 'DeWet' the rooting-cofactors chlorogenic acid and phloroglucinol at the concentration of 100µg l^{-1} yielded 77 % and 81 % rooting, respectively while quercitin and rutinin gave only 46 and 44 % rooting for all cultivars. The basal portion of the cutting rooted better than the terminal portion (78 % versus 58 %). Rooting percentage differed in all treatments in response to IBA application and rooting-cofactors 74 % for 'Florida Prince',62 % for 'DeWet' and 54 % for 'Earlibelle'. The use of IBA and Ca-EDTA proved to be beneficial for rooting of 'Florida Prince'

cuttings and resulted in a rooting percentage of 86 % in wounded hardwood cuttings of this cultivar. It was also shown that the simple sugars glucose, fructose and sucrose as well as the sugar alcohols sorbitol and mannitol accumulated at the base of the cutting during adventitious root formation if the cutting bases were treated with 1000 mg l^{-1} IBA. The concentration of these sugars and sugar alcohols were lower in untreated cuttings compared to IBA treated cuttings during the adventitious root formation process. Studies on the origin of adventitious root formation in stem cuttings of peaches were conducted using light microscopy and transmission electron microscopy. These studies revealed that adventitious roots originate (in peaches) in the vicinity of the vascular bundle tissue and in the cells around them. It was found, however, that adventitious root formation in IBA treated cuttings is associated with the formation of root primordia. These may trigger root initiation and ultimately the development of adventitious roots.

In embryoculture studies the highest number of roots and greatest length of roots per embryo as well as the highest number of embryos forming roots was achieved when either the medium of Murashige and Skoog (MS) (1962) or of Steward and Hsu (SH) (1978) were employed. The Woody Plant medium (Lloyd and McCowan, 1978) and the medium after Schenk and Hildebrandt (1972) were found to be less effective when compared to MS and SH media. Addition of GA_3 (0.01 mg l^{-1}), BAP (0.2 mg l^{-1}) or GA_3 (0.01 mg l^{-1})+ BAP (0.2 mg l^{-1})+IBA (0.5 mg l^{-1})to the media gave the best results with respect to embryos forming roots (86 %), number of roots per embryo (8.0) and total length of roots per embryo (7.57 cm). Furthermore, the following conditions for optimal rooting of peach embryos were established: pH 5.2, 1.5 g l^{-1} agar, 60 g l^{-1} sucrose and 16h light/8h darkness photoperiod. Further studies on the influence of the stratification temperature on germination of embryos revealed the highest germination percentage after exposure to 4°C ±2 constantly for 30 to 60 days. However, this chilling requirement can be successfully substituted by the addition of GA_3, kinetin, zeatin, BA and thiourea to the culture media.

DECLARATION

I hereby declare that the research work reported in this dissertation is the result of my own investigation, except where acknowledged.

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I certify that the above statement is correct.

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LIST OF ABBREVIATIONS

ABA:	Abscisic acid
AR:	Adventitious Rooting
ARF:	Adventitious Root Formation
b:	Basal position of cutting
BA:	Benzyladenine
BAP:	6-benzylaminopurine acid
CHL:	Chlorogenic acid
cv:	Cultivar
CV%:	Coefficient of variation
dw:	Dry weight
EDTA:	Ethylene-diamine tetra acetic acid
fe-EDTA:	Eron ethylene-diamine tetra acetic acid
fw:	Fresh weight
GA:	Gibberellin
GA_3:	Gibberellic acid
IAA:	Indole-3-acetic-acid
IBA:	Indole-butyric acid
JA:	Jasmonic acid
LM:	Light microscopy
LSD (5 %):	Least significant difference
MS:	Murashige and Skoog Medium
NAA:	1-Naphthalene acetic acid
Na-EDTA	Sodium-ethylene-diamine tetra acetic acid
PC:	Cutting position
PHL:	Phloroglucinol
Que:	Quercitin
RC:	Rooting-cofactor
Rut:	Rutinin
± SE:	Standard error
SH:	Steward and Hsu Medium
Shives:	Schenk and Hildebrandt Medium
TEM:	Transmission Electron Microscopy
te:	Terminal cutting position
WP:	Woody Plant Medium

APPENDICES

CHAPTER ONE

General Introduction

1.1 World peach production

Peach, *Prunus persica* (L.) Batsch., is usually cultivated between 30° and 40° latitude, in regions where winters are cold and summers warm. Exceptions to this general distribution pattern occur where microclimate effects of altitude or large bodies of water are found (Childers and Sherman, 1988). Fideghelli *et al.* (1998) indicated that among the deciduous fruit industries, the peach industry is probably the most dynamic in terms of breeding activity, frequently releasing new cultivars and new fruit types (e.g. peaches and nectarines, white and yellow flesh cultivars, freestone and clingstone cultivars, cold tolerant and less cold tolerant cultivars).

The world peach industry produces approximately 11.3 million tons per annum comparatively less than apple industry which produces 28.6 million tons per annum (FAO, 1999). World-wide peach production increased up to 1994 and remained constant thereafter. Geographically, a high concentration of peach production is found in Asia (e.g. China and Korean Republic), Europe (e.g. Bulgaria, Greece, Italy, France) and North and Central America (e.g. Mexico, United States of America, Canada) (FAO, 1999).

Between 1990 and 1996, almost 500 new commercial cultivars were released world-wide. These cultivars were mainly freestone types for the fresh market (57 %), nectarines (34 %) and canning clingstone types (9 %), mostly yellow-fleshed (Fideghelli *et al.*, 1998).

1.2 Conventional peach propagation

The ability to form adventitious roots is a prerequisite to successful peach cutting propagation (Avery and Beyl, 1991). Peach trees perform best when the scion cultivar is grafted onto peach rootstock than when other related species do not perform well when used as rootstocks (Couvillion and Erez,1980). Establishing peach orchards from direct rooted cuttings has been suggested as a way of reducing the initial cost of high-density orchards (Krew and Coston,1983). Direct rooting of peaches has been achieved using hardwood cuttings (Erez and Yablowitz, 1981), semi-hardwood cuttings (Couvillion and Erez,1980) and softwood cuttings (Alward,1984).

Commercial propagators use technologies such as intermittent misting and fogging as well as temperature and light manipulation to alter environmental conditions to maximize rooting percentage (Hartmann *et al.*, 1997).

Differences exist in the rooting ability of cuttings between species and cultivars. Stem cuttings of some cultivars and species (such as *Rosa* and *Hedera helix*) root readily in a mistbed. Cuttings of many difficult-to-root cultivars can only be successfully propagated if specific factors are maintained at optimum level (Libby *et al.*, 1972). Careful selection of cutting material from stock plants and management of cuttings during the rooting process are essential for success.

1.2.1 Factors influencing rooting of cuttings

1.2.1.1 Cutting selection criteria

The type of cutting materials can range from leafy softwood terminal shoots of current growth to dormant hardwood shoots (Hartmann *et al.*, 1997). Some difficult-to-root species are best propagated

from leafy softwood shoots. Such softwood cuttings are known to have a moderate light requirement, since photosynthesis is essential to produce substances which will enhance rooting (MacKenzie *et al.*, 1986). Furthermore, they easily desiccate and can only be propagated successfully by fog or mist systems. Dormant hardwood cuttings in contrast root without actively phosynthesising, as they contain starch reserves. Hence they can be propagated under low light, without mist or under "less critical" mist regimes (Wilson, 1994).

Deciduous species can be propagated when shoots are at different phases of the annual cycle. Hardwood cuttings are taken during the dormant season. Leafy softwood and semi-hardwood cuttings are prepared during the growing season while shoots are still succulent and non-dormant. The time of the year in which the cuttings are taken, plays an important role the in rooting potential (Harrison-Murray, 1991). Many species, display an optimal period for adventitious root formation (Anand and Herberlin, 1975). Softwood cuttings of many deciduous woody species, e.g. *Prunus avium,* taken in spring or summer usually root more readily than hardwood cuttings produced in winter (Hartmann *et al.*, 1997). The effect of the stage of shoot development in the annual growth cycle can also be observed in difficult-to-root deciduous *Rhododendron vaseyi* cuttings, which root readily when taken from summer succulent growth but by late spring rooting ability declines rapidly (Davies, 1984). To determine the most appropriate time to collect cuttings the following parameters can be used: calendar days, days from budbreak, indicator plants and morphological condition of the stock plant (Dunn *et al.*, 1996). Such timing of cutting preparation considers the effect of environmental conditions on shoot development. If hardwood cuttings are planted in early spring, after completion of endo dormancy the rooting potential is very low, as buds open quickly with the

onset of warm days. The newly developing leaves will also transpire heavily, removing moisture from the cutting as well as depleting the cutting of resources.

1.2.1.2 Wounding

Wounding a cutting initiates a chemical signal that induces changes in the metabolism of affected cells (Wilson and van Staden, 1990). These cells at the base of the cutting display enhanced receptivity to respond to auxin and other morphogens (non-auxin endogenous compounds essential for rooting) (Wilson, 1994). The wounding technique involves stripping the cutting of all the basal leaves and leaving only three to four terminal leaves. Removal of these basal leaves allow for air circulation in the area between the retained leaves and the rooting medium. This ultimately leads to reduced leaf drop and disease incidence. A three mm long section of the bark is removed from one side of the cutting base before the cuttings are dipped into an auxin solution (Couvillion and Erez, 1980). Basal wounding has been found beneficial in rooting of cuttings of *Rhododendron canadiens* and *Juniperus* species (Lovell and White, 1986). After wounding, callus production and root initials often increase to a greater extend along the margins of the wound. Wounding a tissue seems to stimulate cell division and production of root primordia (MacKenzie *et al.*, 1986). This is possibly due to an accumulation of auxins and carbohydrates in the wounded area resulting in a new 'sink'. Krishnamoorthy (1970) further stated that tissue injured by wounding produces ethylene, which may indirectly promote adventitious root formation. Wounding cuttings at the base also permits greater absorption of growth regulators applied to that area (MacKenzie *et al.*, 1986).

1.2.2 Anatomical basis of adventitious root formation

The origin of roots in plants differs. The primary root system originates from the radicle development during embryogenesis (Barlow, 1986). The adventitious root system on the other hand initiates endogenously, from tissues within the plant (Sinnot, 1961). Roots which arise from the primary root system (radicle) in an acropetal sequence are characterized by the appearance of further lateral roots as a normal part of development, and are sometimes also called adventitious. According to Barlow (1986), the term adventitious can also be applied to such roots to distinguish them from roots of shoot origin.

Various plant species are able to form adventitious roots naturally, e.g. *Zea mays* and *Arachis hypogaea*. Adventitious roots can be divided into two categories: preformed roots and wound-induced roots. Preformed root initials and primordia develop naturally while the plant part on which they develop is still attached to the parent plant (Avery and Beyl, 1991). This type of adventitious root normally lies dormant until the stems are made into cuttings and placed under environmental conditions favorable for further development and emergence of the primordia as adventitious roots (Lovell and White, 1986). Wound-induced roots can develop after the cutting has been severed from the mother plant. They are formed *de novo* after the wounding incident (Davies *et al.*, 1982). Every time living cells are injured by a cut and exposed to air (e.g. when producing stem cuttings), a response to this wounding is initiated (Cline and Neely, 1983). These include, dying of the outer injured cells, formation of a necrotic plate and sealing of the wound with suberin. The living cells behind the necrotic plate divide normally and a layer of parenchyma cells (callus) near meristems develop into a new meristem out of which a parenchyma layer is formed. Lastly, the cells in the

vicinity of the vascular cambium and the phloem begin to divide and initiate adventitious roots *de novo* (Hartmann *et al.*, 1997).

Further developmental changes occurring chronologically in *de novo* adventitious root formation (ARF) include: dedifferentiation of specific differentiated cells, formation of root initials from cells near vascular bundles or vascular tissues, development of root initials into organized root primordia, growth and emergence of the root primordia outward through other stem tissues, and lastly, formation of vascular tissue between the root primordia and the vascular tissues of the cutting (Avery and Beyl, 1991). However, the origin of adventitious roots in peaches has also been associated with cells surrounding the vascular bundles, but this origin seems to be cultivar dependent (Arafeh *et al.*, 1991).

In herbaceous and woody plants adventitious roots usually originate in the vicinity of vascular bundles (Preece, 1993), but the tissues of origin can vary widely depending on species and propagation technique (Altamura *et al.*, 1991). While adventitious roots arise in the phloem parenchyma in *Lycopersicon esculentum*, *Cucurbita maxima* and *Vigna radiata*, in *Crassula* species (*arborescens* and *ovata*) they arise in the epidermis (Blazich and Heuser, 1979). Adventitious roots in stem cuttings of woody perennial plants usually originate from living parenchyma cells in the young, secondary phloem, but sometimes also in the vascular rays, cambium, primary phloem, callus, or lenticels (Hartmann *et al.*, 1997).

1.2.3 Biochemical basis of adventitious root formation

1.2.3.1 Endogenous rooting inhibitors

Endogenous inhibitors can retard rooting in certain plant species. In *Vitis vinifera* cvs, dipping

cuttings in water for 24 hours leaks inhibitors out of the cutting and increases the rooting percentage (Wood and Cameroon, 1989). Difficult-to-root hardwood cuttings of *Tabernae montana divaricata* contain a cinnamic acid derivative which inhibits rooting, while no detectable amount of this phenolic compound is formed in easy-to-root softwood cuttings of the same species (Cuir *et al.*, 1993). Cuttings of difficult-to-root mature *Eucalyptus* species (Crow *et al.*, 1971), *Castanea sativa* (Vietez *et al.*, 1987) as well as *Dahlia* cultivars (Biran and Halevy, 1973) have a higher concentration of rooting inhibitors than easy-to-root juvenile forms of *Hedera helix* and *Hibiscus rosa-sinensis*.

1.2.3.2 Rooting co-factors (auxin synergists)

Rooting co-factors are naturally occurring substances that appear to act synergistically with indoleacetic acid (IAA) in promoting rooting (Kling *et al.*, 1988). The rooting co-factors extracted form *Hedera helix* represent a group of oxygenated terpenoids as well as isochlorogenic acid. These phenolic compounds react synergistically with IAA in root production in the *Vigna radiata* bioassay (Fernqvist, 1966). Rooting co-factors have also been found in *Acer* species (Kling *et al.*, 1988). Fadl and Hartmann (1967) isolated an unidentified endogenous root-promoting factor from basal sections of hardwood cuttings of the easily-rooted *Pyrus communis* cultivar 'Old Home'. Extracts of the difficult-to-root *Pyrus communis* cv 'Bartlett', however, did not obtain s this root-promoting factor.

Rooting co-factors and inhibitors have been studied in detail to understand rooting phenomena. Rooting co-factors have been correlated to a positive rooting responses, though no cause-effect relationship has been established. Bhattachrya (1988) proposed that the predisposition of cells to initiate root primordia depends on the activity of certain enzymes. However, auxin-phenol-enzyme

complexes have not been found *in vivo*, and promoter-inhibitor systems of rooting have not been universally observed in plants (Bassuk *et al.*, 1981).

1.2.4 Plant growth regulators and ARF

The main classes of plant growth regulators (auxins, cytokinins, gibberellins, ethylene and abscisic acid), as well as other substances, such as polyamines and phenolics, take part in root initiation either directly or indirectly (Davis and Haissig, 1990). Plant growth regulators influence various aspects of root growth and development and any number or combination of these may influence root formation; but, in most cases, the primary trigger initiating root development is auxin (Scagel and Linderman, 2000). Furthermore auxins are the most commonly used substances applied to enhance rooting in cuttings. Indole-3-acetic acid (IAA) is the most common natural form of auxin found in plants. The response of plants to changes in IAA levels is dependent on the absolute concentration, on the amount relative to other plant growth substances and on tissue sensitivity (Hartmann, *et al.*, 1997). The effect of exogenously applied auxins on root development has been variable. Kelly and Moser (1983) found that root application of IBA to *Liriodendron tulipifera* increased rooting percentage in both spring and autumn cuttings. Specific responses in root growth and tree survival of *Pinus contorta* and *Picea engelmannia* to application of auxin (applied as an IBA solution) have been reported (Scagel and Linderman, 2000). IAA is not used commercially for the stimulation of rooting in cuttings because it is sensitive to bacterial destruction in unsterilized solutions. It is also sensitive to in activation by light (Strydom and Hartmann, 1960).

1.2.5 Correlative effects: hormonal control of adventitious root and bud formation

The existence of a specific root-forming factor was first postulated by Went in 1929. He found that leaf extracts from certain *Acalypha* species (*gracilens* and *gray*) applied to *other Acalypha* or to certain *Carica* species (*papaya* and *paper*) induced root formation in vitro (Went, 1934). The formation of adventitious roots in stems has been explained by the basipetal movement of root initiating substances from the leaves to the roots. In extending this concept, it was postulated that a specific root forming substance manufactured in the leaves, moves downward to the base of the stem and promotes root formation in cuttings (Sachs *et al.*, 1964). It was further shown that sprouting buds in cuttings of *Salix spp.*, *Populus spp.*, *Ribes nigrum* and *Vitis vinifera* promote the development of adventitious roots just below the buds. Howard (1965) assumed that plant growth regulators are formed in the developing buds and transported via the phloem to the base of the cutting, where they stimulate ARF.

For the initiation of adventitious roots, the presence of an actively growing shoot tip (or lateral bud) is necessary during the first three or four days after the cuttings are taken (Haissig and Davis, 1994). However, after the fourth day the shoot terminal and auxiliary buds can be removed without interfering with subsequent root formation. Went (1934) showed in *Pisum sativum* that the presence of at least one bud on the pea cuttings is essential for root production.

The presence of leaves on cuttings exerts a strong stimulating influence on rooting. The stimulatory effect of leaves on rooting of stem cuttings has been shown in studies with *Persea americana* (Reuveni and Raviv, 1981). Leaves and buds produce auxins and the effects of the polar basipetal transport of auxins can, according to Haissig and Davis (1994), be observed at the base of cuttings.

Absence of leaves in hardwood cuttings can therefore be assumed as one reason why these types of cuttings take longer to root than softwood cuttings with leaves.

1.3 Peach propagation by embryo culture

1.3.1 Seed embryogenesis

Seed formation involves three independent developmental stages: embryogenesis, seed development and onset of dormancy (Fosket, 1994). In the majority of seeds embryogenesis and seed development can be divided into five stages. Stages one to three are named globular, heart, and torpedo stage respectively. These first three stages are, in most species (including peach), completed within 30 days after fertilization (Fosket, 1994).

In the globular stage the eight central cells divide longitudinally and then transversely yielding 32 central cells in addition to the 32 protodermal cells produced 60 hours after fertilization. The globular phase of plant embryogenesis terminates when localized, rapid cell divisions occur, leading to the formation of the two cotyledon primordia. Growth of the cotyledon primordia gives the embryo the heart shape (heart-stage embryo). A major event of the heart stage is the establishment of axial polarity. In stage three, the torpedo stage, hypocotyl and radicle can be recognized and the vascular tissue begins to differentiate within these organs. The bulk of the stored proteins is synthesized and deposited in the cotyledon cells during stage four, the midmaturation phase. At the end of the maturation phase the seed usually enters into the dormancy phase which is characterized by transcription of genes and stop of protein synthesis followed by slight dehydration of the embryo (Fosket, 1994).

1.3.2 Seed dormancy

Seeds of most temperate trees and shrubs, even though mature, will not germinate unless they have been chilled for a certain amount of time (Westwood, 1990). Seed dormancy describes the inability of seeds to germinate although environmental conditions are favourable (Mathew and Karikari, 1990). Seed dormancy may be due to physical or physiological factors. This classification as well as the currently used terminology of endodormany, paradormancy, and ecodormancy is based on an interpretation of Samish (1954) and Romberger (1963). The initial reaction prescribed by this dormancy classification ultimately leads to growth, i.e. the first step in the chain reaction that may be manipulated to alter the expected growth response, provides a relatively convenient and physiologically meaningful base for classification (Lang *et al.*, 1987).

According to Samish (1954) ecodormancy is a dormancy due to one or more unsuitable environmental factors that are generally non-specific in their effect on overall plant metabolism. For example, water or nutrient deficiencies or extremes of temperature have a broad effect on plant growth and function, not a precise regulatory effect, limited to a specific meristematic or receptive structure. In a broad sense, one might mistakenly use ecodormancy to include all environmental factors associated with dormancy (Salisbury, 1986). Likewise one might mistakenly use the term endodormancy to include all types of dormancy, since all cases eventually are manifested through internal factors (Aue *et al.*, 1999). However, the above definitions clearly distinguish between specific physiological responses to precise environmental conditions (such as chilling or photoperiodic induction) and non-specific physiological responses to generally unfavorable environmental factors (such as low oxygen, drought, or high temperature). Ecodormancy only involves the absence of certain environmental conditions that are an absolute requirement for general

growth by all plants (Lang *et al.*, 1987). Ecodormancy may be thought of as a mere pause in growth until all these basic factors are adequate. The currently used dormancy terminology does not describe developmental phases, but rather the processes that constitute the observed phases. As such, they are as readily applicable to buds as to seeds and bulbs (Salisbury, 1986). Endodormancy describes the phenomena where the initial reactions leading to dormancy are specific perceptions of an environmental or endogenous signal within the affected structure alone. Paradormancy involves a specific biochemical signal originating in a structure other than the affected structure as the initial reaction (Amen, 1968). The biochemical signal may be environmentally triggered or may not be of environmental origin, such as the continuous inhibition of visible growth of lateral buds (apical dominance) by morphogenic factors produced in nearby organs (apices, subtending leaves, testae, etc.) (Lang *et al.*, 1987).

Zigas and Coombe's (1977) study of peach seedlings revealed a bifunctional role of stratification in seed germination. Germination was found to be inhibited by the seed coat (ecodormancy). Removal of the seed coat allowed germination but does not result in epicotyl elongation. Exposure of dormant peach seeds to low temperatures is necessary to fulfill the chilling requirement for breaking dormancy to allow germination (Scalabrelli and Couvillon, 1986).

Two physiological processes which occur at the end of the growing season, cold acclimation and development of dormancy, are superimposed onto each other. Although they are integral parts of the annual cycle of woody perennials, the understanding of these processes is poor (Dennis, 1974). Arora *et al.* (1997) postulated that the onset of gene expression resulting in protein formation is

correlated with chilling requirement and the development of dormancy.

The peach fruit can serve as a model for studying growth related changes in seeds and buds and their relationships with certain physiological processes because its growth occurs in four well defined development stages (seed development, fruit set, fruit growth and fruit maturity) (Valpuesta *et al.*, 1989). The seed plays a critical role in growth and viability of the fruit growth during stage I of fruit development (Taylour *et al.*,1984). Plant growth regulators have been associated with different stages of fruit growth in peaches. Changes in indoleacetic acid (Aue *et al.*, 1999) and peroxidase activities (Valpuesta *et al.*, 1989) have been determined and correlated to fruit growth. Dormancy in peach seeds can be associated with certain levels of stimulating or inhibiting substances, amounts of active phytochrome, various physical regimes, or a combination of these factors (Taylorson and Hendricks, 1979).

1.3.3 Hormonal and environmental control of seed dormancy

Wareing and Saunders (1971) stressed the importance of covering structures, such as bud scales and seed coats in inhibiting organ development, and postulated that dormancy is due to the accumulation of products of anaerobiosis. A modified form of this theory has been put forward more recently by Hilhorst and Karssen (1992). Although other factors are clearly also involved, there is growing evidence that hormones play a paramount role in many aspects of seed and fruit growth and development, which stresses the likelihood that hormones have an important function in controlling seed dormancy (Toorop *et al.*, 1996).

There are various analogies between bud dormancy and certain forms of seed dormancy, particularly

where seeds exhibit a chilling requirement for germination (Wareing and Saunders, 1971). Moreover, comparing dormancy in buds and seeds of a single species, such as birch *(Betula pubescens*), shows that dormancy of both organ types can be overcome either by chilling or a combination of exposure to long photoperiods and endogenous biosynthesis of gibberellic acid (Weges, 1991). Although the embryo itself is dormant in a number of species, especially in those having seeds with a chilling requirement (e.g. *Pyrus communis*, *Malus domestica*), in many others the embryo is not dormant and dormancy is displayed only by the intact seed (Bukovac, 1988). In such cases, which are normally referred to as seed coat imposed dormancy, the seed coat exerts the inhibitory effect on embryo germination (Couvillon and Erez, 1985).

Thus, chilling overcomes both embryo and seed coat imposed dormancy in temperate zone fruit trees. Prominent among theories explaining dormancy due to plant growth regulators is the involvement of specific germination inhibitors in seed dormancy. The idea that inhibitors may be responsible for preventing premature germination of the seeds in succulent fruit was put forward by Wareing and Saunders (1971), and the presence of germination inhibiting substances (e.g. ABA), was subsequently demonstrated for a number of succulent and non-succulent fruit (Balandier *et al.*, 1993).

The cultivation and domestication of grain species, such as maize (*Zea mays)* and barley (*Hordeum vulgare* L.), as well as of vegetables, has undoubtedly included selection for sufficient primary dormancy (Lang *et al.*, 1987). Seeds of most plants display primary dormancy when separated from the mother plant. This not only prevents immediate germination but also regulates the time, condition and place when germination will occur (Atwater, 1980). In nature, different types of primary dormancy have evolved to aid survival of species (Toorop *et al.*, 1996) by programming the

time of germination for particularly favourable times in the annual seasonal cycle. Secondary dormancy is a further survival mechanism that can be induced under unfavorable environmental conditions and may further delay the onset of germination (Karssen, 1980). Secondary dormancy has been defined by Bewley and Black (1994), as an 'adaptation to prevent germination of an imbibed seed if other environmental conditions are not favourable'. Such unfavourable conditions imposing secondary dormancy range from too high and too low temperatures to prolonged darkness. Knowledge of the ecological characteristics of the natural habitat of a species can aid in establishing causes of dormancy and hence help to develop treatments to induce germination (Willemsen, 1975).

1.3.4 Plant growth regulators and seed dormancy

Seed dormancy has only been studied in very few species which exhibit a chilling requirement. It has been found that embryo dormancy of the temperate-zone forest tree *Fraxinus excelsior* can be overcome by exposure of the seeds to low temperatures for varying lengths of time (Lang *et al.*, 1987). Results obtained by Lang *et al.* (1987) indicate that seed dormancy in *Fraxinus excelsior* is controlled by a balance between endogenous growth inhibitors, especially ABA, and promoters, particularly gibberellins. For several types of dormancy ABA has been shown to be the main component of the inhibitory fraction (Balandier *et al.*, 1993).

1.3.5 Plant growth regulators application and seed dormancy

Experimental evidence supports the concept that specific endogenous growth promoting and inhibiting compounds are directly involved in the control of seed development, seed dormancy and germination (Black, 1991). Furthermore, different concentrations of various plant growth regulators trigger specific responses in seed development. Therefore, plant growth regulators have been

correlated to the metabolic activity of the seed (Toorop *et al.*, 1996).

Several studies have been carried out to investigate the effects of plant growth regulator application particularly of gibberellins and cytokinins, on seed germination (Wareing and Saunders, 1971). Addition of these substances will bring about germination of a wide range of species exhibiting various forms of seed dormancy (Lang *et al.*, 1987) including dormancy due to non-fulfillment of the chilling requirement as well as the need for an after-ripening period in dry storage.

1.3.5.1 Gibberellins

Gibberellins are a group class of hormones involved in promoting seed germination (Hartmann *et al.*, 1997). Gibberellins appear not to be involved in the control of dormancy *per se* but rather are important in the promotion and maintenance of germination, i.e., they act after ABA-mediated inhibition of germination has been overcome (Leon-Kloosterziel *et al.*, 1996). Gibberellins are also known to reduce the requirement of seeds for light and counteract the inhibitory effects of ABA, frequently in combination with cytokinins (Bewley and Black, 1994). While GA_3 is effective in a large number of species, GA_4 and GA_7 are even more active at lower concentrations than GA_3 in some species (Wareing and Saunders, 1971).

1.3.5.2 Auxins

Auxins are essential in many plant growth processes, such as stem growth, adventitious root formation, lateral bud inhibition, abscission of leaves and fruits and activation of cambial cells (Hartmann *et al.*, 1997). The most common and naturally occurring is indole-3-acetic acid (IAA). IAA has been found to promote rooting on stem segments and root formation in cuttings. There are,

however, other synthetic auxins such as indolebutyric acid (IBA) and napthaleneacetic acid (NAA) which have a higher auxin activity than IAA. These compounds are widely used to root stem cuttings and their addition to a culture media results in root formation in microcuttings (Hillman and Gaston, 1961).

1.3.5.3 Ethylene

Ethylene stimulates germination of certain seeds (Fosket, 1994). The first report of an effect of ethylene on seed germination was that of Nord and Weicherz in 1929, when they demonstrated that ethylene and acetylene increased the rate of germination and growth of *Hordeum vulgare* (Abeles, 1973). It was observed that the promotion of germination was not a specific ethylene response, since carbon dioxide had a similar effect, and the combination of carbon dioxide and ethylene resulted in the highest germination rate (Eplee, 1975). However, some studies suggest that ethylene may be involved in relieving seed dormancy in some plants (Bewly and Black, 1994). Ethylene is produced by germinating seeds of various species and treatment of dormant seeds with ethylene induces a higher percentage of germination in such seeds than germinating them in an ethylene-free environment (Bleasdale, 1973).

1.3.5.4 Cytokinins

Evidence for the involvement of cytokinins in seed germination is based on experiments where cytokinin application stimulated bud growth as well as seed germination. The cytokinin concentration measured within the relevant tissues correlates to bud growth as well as to seed germination (Powell, 1987). Cytokinin activity tends to be high in developing fruits and seeds, but decreases and becomes difficult to detect as these structures mature. In seed germination, cytokinins are believed to offset

the effect of the inhibitors, especially ABA (Hartmann *et al.*, 1997). Cytokinins have been described as playing a 'facilitative' role in germination in allowing gibberellic acid to function (Bewly and Black, 1994).

1.3.5.5 Abscisic acid (ABA)

The differential sensitivity of embryos to ABA may be important to maintain dormancy of mature seeds (Hilhorst, 1995). ABA is a naturally occurring compound important in seed germination and plant growth in general (Bewley and Black, 1994). It plays a role in "precocious germination" of the developing embryo in the ovule (Finkelstein and Crouch, 1987). ABA also tends to increase with fruit maturity and prevent vivipary by inducing primary dormancy (Toorop *et al.*, 1996). ABA has been isolated fro seed coats of dormant *Prunus persica, Juglans regia, Malus domestica, Rosa canina* and *Prunus martima*. The concentration of ABA in the seed coat, however, decreases during stratification (Walton, 1980)

1.4 Mechanism of dormancy control by hormones

Dormancy of buds and seeds involves an internal block to growth. Furthermore dormant tissue has a lower rate of metabolism than actively growing tissue (Wareing and Saunders, 1971). In the 1980s it was believed that differences in the respiratory activity are responsible for organs being in a dormant or in a non-dormant state. Since then, advances in molecular biology have led to different opinions, with attention now focussed on DNA and RNA metabolism in dormant and non-dormant organs (Aue *et al.*, 1999). This approach is consistent with the hypothesis that dormancy is under hormonal control, in view of considerable evidence that hormones have marked effects on nucleic

acid metabolism (Bleasdale, 1973).

ABA application has been shown to inhibit RNA and DNA synthesis in a variety of plant tissues including seeds (Ross, 1983). ABA inhibits $^{32}PO_4^{-2}$ incorporation into r-RNA, t- RNA and DNA in *Phaseolus vulgaris* embryos. Huang and Powell (1981) observed that GA_3 stimulates the germination of lettuce seeds when DNA synthesis is inhibited by pre-irradiation with ^{60}Co gamma rays, and that ABA inhibits this effect of GA_3.

The primary mode of action of GA_3 by which it stimulates RNA synthesis in various plants is unknown, but the well known effect of GA_3 on α-amylase synthesis in *Hordeum* vulgare endosperm (Walker and Dumbbroff, 1981) strongly suggests that it has an important role in nucleic acid metabolism and/ or protein synthesis. Seemingly gibberellins and ABA play opposing roles in cereal grain development supporting evidence comes from findings that the gibberellin induction of the α-amylase genes can be inhibited by ABA (Lanahan *et al.*, 1992).

Hormones influence almost every aspect of plant growth and development (Fosket, 1994). The most accepted theory of the mechanism of plant hormone action is derived from well-characterized animal hormone models. According to Guiltinan and Diekman (1994), plant hormones have to be perceived by a hormone receptor, which will initiate a signal transduction sequence, and one target of signal transduction will be gene expression.

These authors postulated the following mechanism of hormone action:

Hormone →Receptor → Signal Transduction → Gene Expression → Effect on Plant Growth and Development.

Many of the physiological effects of plant hormones are brought about by alterations in gene expression. The expression levels of hormonally regulated promoters are thought to be controlled via interactions with DNA binding proteins (transcription factors).

A large number of genes induced by ABA have been identified (Walker-Simmons, 1987). ABA is known to have a particular effect during seed maturation and during osmotic responses. It has been identified as the substance that prevents *Triticum durum* embryos from germination and promotes maturation when immature embryos are cultured in vitro through effects on gene regulation (Quatrano *et al.*, 1993).

The effects of cytokinins on gene expression have been previously reviewed (Chen, 1989). Genes regulated by cytokinins include those triggering chlorophyll a/b synthesis (Flores and Tobin, 1988), ribosomal genes and several other *Glycine max* genes of unknown function (Crowell *et al.*, 1990). Auxins have been found to be involved both in 'down' and 'up' regulation of genes detectable after long-term auxin treatment and persist for many hours, particularly in dividing tissues (Van der Zaal *et al.*, 1991). The α- amylase genes in cereal grains have been shown to be regulated by gibberellins. The nuclear factors involved in this control have been identified (Huttly *et al.*, 1992). Therefore efficient use of plant growth regulators requires an understanding of how these act and interact to optimize plant propagation either from seeds or cuttings.

1.5 Problem Statement

Methods that optimize propagation of *Prunus persica* would provide the much needed basis for increasing the productivity of seedlings produced from embryo or tissue culture and would increase rooting of cuttings and could ultimately improve the establishment and survival of peach trees in the

orchard. Although the use of cuttings as a means of propagation has long been practised, studies that optimize rooting percentages and survival of such cuttings have been limited. Embryoculture and organ culture have also been used as alternative means of propagating *Prunus* species, although their use at commercial level has been limited due to lack of research. Plant growth regulators such as auxins, gibberellins, cytokinins and ethylene have successfully been used in micropropagation of *Prunus* species in an attempt to alleviate the chilling requirement, but their use has been overlooked in commercial production of *Prunus* rootstocks. Methods that can lead to the optimization of propagation of *Prunus persica* by increasing the germination of embryos cultured *in vitro* or increasing the rooting of cuttings can set the basis through which future rootstocks of high compatibility with desired scions can be produced and selected. Hence, the development of propagation techniques to optimize propagation methods in peaches is seen as an important step in bringing the knowledge and manipulation of *Prunus persica* up to the level similar to that of certain other perennial fruit crop species.

1.6 Objectives

The aims of the research work reported here were to optimize peach embryo and cutting propagation. Peach embryo culture was used as a method to obtain the highest possible germination, emergence rate and survival percentage. Optimizing cutting propagation as well as embryo culture had the aim to establish a quicker and more efficient peach rootstock production. With this in mind the following aspects were studied:

a. Rootability and survival of peach cuttings propagated from softwood, semi-hardwood and hardwood cuttings

b. Anatomy of adventitious root formation in peach hardwood cuttings

c. Effect of stratification in germination and emergence of peach seed embryos

d. Optimization of peach embryoculture methodology with respect to culture media and media constituents

CHAPTER TWO

Effect of IBA and other ARF treatments promoting rooting on softwood, semi-hardwood and hardwood cuttings of three peach cultivars

2.1 Introduction

Cuttings prepared from soft, succulent spring growth of deciduous or evergreen species are classified as softwood cuttings (Hartmann *et al.*, 1997). The softwood stage of a shoot ranges from two to eight weeks after bud break. Softwood is produced during growth flushes which in temperate climates occur mainly during spring (Marini, 1983). As the wood matures from soft to semi-hardwood to hardwood, cuttings become hardier to frost. Furthermore in many species, including peach, softwood cuttings generally root easier than semi-hardwood and hardwood cuttings, but require more sophisticated propagating conditions (Okie, 1984). Softwood cuttings are always prepared as leafy cuttings, they consequently have to be handled more carefully to prevent desiccation and must be rooted under conditions such as intermittent mist which will avoid excessive water loss from the leaves. The temperature of the rooting medium for peaches should be maintained between 23° and 27°C (Okie, 1984). Softwood cuttings produce roots in two to five weeks and generally respond better to auxin application than other cutting types. Hence, a relatively low auxin concentration can stimulate ARF. Semi-hardwood cuttings, on the other hand, are taken from woody, leafy summer and early autumn cuttings of deciduous plants and are usually 7.5 to 15 cm long shoot with the leaves still attached and consist of partially matured wood (Couvillon and Erez, 1980). Both basal and terminal shoot regions can be used as cutting material but the basal parts will usually root better (Okie, 1984). It is necessary to root leafy semi-hardwood cuttings under

conditions that will keep water loss from the leaves minimal; hence, commercially they are rooted under intermittent mist (Bollmark and Eliasson, 1986).

Hardwood cuttings are made from mature, firm, dormant wood after the leaves have abscised (Hartmann *et al.*, 1997). Propagation by hardwood cuttings is one of the least expensive and oldest methods of vegetative propagation. Hardwood cuttings are prepared during the dormant season in late autumn, winter or early spring, usually from the previous seasons growth (Okie, 1984). Synthetic root-promoting chemicals that have been found most reliable in stimulating adventitious root production in either softwood, semi-hardwood or hardwood cuttings are the auxins, IBA and NAA (Hartmann *et al.*, 1997). IBA is more widely and commonly used than NAA because of its non-toxicity to plants, even at high concentrations, and its effectiveness in promoting rooting in a large number of plant species (Okie, 1984). Many diverse compounds, which are generally thought to influence auxin levels, have also been shown to influence ARF. Phenolic compounds are regarded as positively important although there is only limited support for this concept from correlative studies of phenolic levels and rooting ability of cuttings (Jarvis, 1986). Phenolic substances such as chlorogenic acid, phloroglucinol, rutinin and quercitin constitute the most important secondary metabolites in plants which can improve adventitious root formation with or without hormones. Jackson and Harney (1970) established a clear correlation between rooting percentage and total phenolics content of woody cuttings. The terms "auxin-synergists" and "rooting co-factors" have been used to describe both, natural and synthetic phenolic compounds that enhance the rooting response of cuttings to applied auxin (Jarvis, 1986; Jackson and Harney, 1970). Rooting co-factors are naturally occurring substances.

The process of ARF is still not fully understood, and it is not clear why cultivars of some species differ in their rooting ability (Ozkaya and Celik, 1999). As endogenous levels of plant hormones seem to represent the influencing ease of ARF, plants have been grouped according to their rooting potential on the basis of hormone levels and types (Bollmark and Eliasson, 1986).

The origin of the shoot material used in producing a cutting, and the time of the year when the cutting is taken, are important factors in rooting peach cuttings (Bassuk and Howard, 1981). However, the interaction of phenol/phytohormone application with origin of shoot material and age and rooting percentage of cuttings has not been evaluated in detail in peaches.

Basal wounding has been found to be beneficial in rooting horticultural species, including, *Actinidia chinensis* (yangtoo) (Sim and Lawes, 1981), *Amelanchier alnifolia* (saskatoon) (Bishop and Nelson, 1980), *Malus domestica* (apple) (Gorecki, 1979) and *Prunus persica* (peach) (Couvillion and Erez, 1980). Basal wounding involves stripping the basal leaves off a cutting (normally three to four basal leaves are removed) and making an incision, extending from the base to a height of three mm on either side of the cutting.. This technique has certain benefits such as reducing the required propagation bench space as cuttings can be placed deep in the mistbed, allows flexibility to work with different size propagules, improves the contact area between the cutting and the media and can potentially improve the absorption of rooting compounds (Edwards and Thomas, 1979). Following wounding, callus production and root development frequently intensify along the margins of the wound. Wounding tissue can furthermore stimulate cell division and the production of root primordia (MacKenzie *et al.*, 1986). Although stem tissue is wounded in the process of making a

cutting, in some species (e.g. *Prunus domestica* (plum), *Juglans regia* (walnut) and *Senecio cineraria* (cineraria)) additional wounding by incising or splitting the base enhances the rooting ability of the cutting (Howard *et al.*, 1984).

Wounding causes biochemical changes that trigger response phenomena in previously quiescent, unwounded cells near the wounded cells which then become metabolically active (Imaseki, 1985). The wound surface, or adjacent wound-affected cells, generate signals which are transmitted through at least several cell layers inducing these quiescent cells to become active. Although the chemical initiation of the signal generated by wounding has been investigated, its cause remains unknown (Howard *et al.*, 1984). One or more plant hormones seem to be involved in a signaling process. Investigations have been carried out to determine the effects of exogenously applied plant hormones on wound reactions at both, physiological and biochemical levels, and changes in plant hormone levels in tissues exposed to wounding have been found to correlate with ARF. Plant hormones are likely to be involved in the regulating wounding response (Mitsuhashi-Kato *et al.*, 1978). Thimann (1972) showed that wounding combined with auxin application positively affects adventitious root formation of stem cuttings. The degree of wounding at the base of the stem will influence the degree of rooting (Thimann, 1972). A longitudinal incision (two to three mm) at the base of pea (*Pisum sativum*) stems increased the number of adventitious roots formed (Thimann, 1977). This phenomenon, probably related to this effect of wounding on root formation, has been investigated in pea epicotyl sections where endogenous phenolic substances, such as caffeic and chlorogenic acid, were found to interact with auxins in stimulating adventitious root formation (Hackett, 1970). Phenolic substances are widely distributed in higher plants, and it is well established that wounding

or any other form of injury greatly stimulates their biosynthesis and accumulation near an injured area (Jarvis 1986). It is thus probable that wound-induced phenolic substances interact with auxin to stimulate root initiation (Imaseki, 1985).

Auxins have been shown to interact with certain nutrients and salt formulations to promote ARF (Bellamine *et al.*, 1998). Such salts include KNO_3, Ca-EDTA (Hartmann *et al.*, 1997), Na-EDTA and Fe-EDTA (Mikesell, 1992). Calcium is found in greater quantities than any other inorganic element in plants, and it increases transpiration flow in the xylem (MacLaughlin and Wimmer, 1999). Ca, Fe or Zn-EDTA can correct deficiency symptoms of micronutrient cations but cannot correct deficiencies in more abundant competitive cations such as magnesium (Evans *et al.*, 1991). The high affinity of calcium salts for anions results in the ability to open microtubuli and tubuli within the xylem tissue which enhances the basipetal/polar transport of auxins. Ca-EDTA and Fe-EDTA have also been found to positively influence regeneration of juvenile explants and callus formation of *Ziziphus mauritiana* and *Ziziphus nummularia*. It has also been reported that an increase of the Fe-EDTA concentration in the medium (from 27.8 to 33.6 mg l^{-1}) promotes bud growth and enhances callusing in these species (Mathur *et al.*, 1993).

The major Ca transporter at the plasma membrane as well as at the ER (Jones *et al.*, 1993) is a calcium pump driven by ATP combined with a Ca^{2+}/H^{+} antiporter (Kasai and Muto 1990). The Ca^{2+} transport at the tonoplast can also be achieved by a Ca^{2+}/H^{+} antiport energized by the proton-motive force of proton-pumping ATPase and phosphatase (Marschner, 1998). The role of calcium in auxin action, especially that of IAA, seems to be very complex and is not clearly understood (Thomas *et*

al., 1994). IAA stimulates the release of calcium from the cytosol into the extracellular space, possibly by activating a plasma membrane calcium pump (Mikesell, 1992). Secondly, IAA may affect calcium ion efflux from the vacuole, across the tonoplast, into the cytosol. The release of calcium from intracellular compartments of animal cells appears to be mediated by inositol triphosphate (IP_3), and a similar mechanism may exist in plant cells. The replacement of calcium ions from the exchange sites in the stem tissues with aluminium (Wallace, 1966) or sodium (Lahaye and Epstein, 1971) allows calcium to move acropetally from the stem tissue to the leaves, creating a sink for other cations at the base of the cutting. It is this acropetal movement of calcium and other cations that is believed to open transport channels through which molecules such as IAA and IBA can be transported basipetally to induce rooting (Taiz and Zeiger, 1991). Due to the inability of calcium to enter membranes of stem tissue and stomatal cells, a chelate has to be used as a carrier to penetrate cell membranes and release calcium into the cytoplasm. This increases the cytosolic calcium, which will ultimately enhance the basipetal movement of auxin to induce callusing and rooting (Taiz and Zeiger, 1991).

ARF is an endergonic process (Jarvis, 1986). Total cutting mass can, however, increase during ARF before the development of a root system (Haissig, 1983). The energy demand to support rooting varies between species and depends, to a certain extent, on the type of cutting. Woody cuttings require weeks or months to root. During this time they can produce large amounts of callus and, hence, undergo a substantial increase in mass (Haissig, 1983). It has been suggested that cuttings root best under conditions which yield a high internal total non-structural carbohydrate (TNC) concentration before and during rooting (Leakey, 1983). The carbohydrate content of cuttings can be influenced by auxin treatments, which may enhance the mobilization of carbohydrates from the

leaves and apical parts of the cutting and increase the auxin transport to the rooting zone (Middleton *et al.*, 1980).

In some species the content of starch, sugars, total carbohydrates or nitrogenous compounds influences rooting (Gill, 1995). Haissig (1984) suggested that total soluble carbohydrate and starch levels in cuttings are positively related to the rooting ability but not through any cause-effect relationship. Total carbohydrates have been reported to equally accumulate in upper and basal stem parts of *Pinus banksiana* cuttings during propagation (Haissig, 1984), but only basal parts formed roots. Veierskov (1988) found that total carbohydrate levels in cuttings may have a stronger correlation to root growth than to root initiation. Furthermore, reducing sugars and sucrose, but not starch, have been reported to differ between upper (non-rooting) and basal (rooting) stems of *Pinus banksiana* cuttings during ARF (Haissig, 1986). Therefore, investigating the breakdown of carbohydrates into a soluble, readily metabolizable form (soluble storage forms) during rooting may aid in determining the roles of carbohydrates in the ARF process (Haissig, 1984). Carbohydrate accumulation in leafy cuttings often occurs first in the basal regions and thereafter in the apical parts, although there are exceptions, e.g. *Prunus domestica*. In *Pisum sativum* carbohydrates accumulate in the basal zones to a specific level and are then redirected to other regions (Davis and Potter, 1981). According to these authors, carbohydrate accumulation does not occur in basal regions of pea cuttings if the basal regions already contains a certain amount of carbohydrate when cuttings were removed from the stock plants. Sucrose, glucose, fructose, sugar alcohols and starch have been most frequently reported to accumulate in cuttings during the ARF process (Davis and Potter, 1981).

In an attempt to optimize rooting in peach cuttings and to determine the best period of preparing the cuttings, the following studies were carried out: The influence of phenolic rooting-cofactors (phloroglucinol, chlorogenic acid quercitin and rutinin), the stimulatory effects of IBA on adventitious root formation and the influence of Ca-EDTA on rooting of different cultivars ('DeWet', 'Earlibelle' and 'Florida Prince') was evaluated over a period of four seasons. Finally, changes of soluble sugars (fructose, glucose and sucrose) and sugar alcohols in peach cuttings were studied in 'Florida Prince' softwood cuttings.

2.2 Materials and Methods

2.2.1 Effect of IBA and rooting-cofactors at various seasons on rooting of cuttings of three peach cultivars

The study was conducted for a period of one year (August 1999 to July 2000) on the early ripening peach cultivars 'DeWet', 'Earlibelle' and 'Florida Prince'. These cultivars can be successfully used as peach rootstocks (Anon, 1990). Shoots were collected at six week intervals as leafy softwood, leafy semi- hardwood and leafless hardwood cuttings from the Ukulinga Research Farm (University of Natal, Pietermaritzburg). Four trees (five years old) of each cultivar were selected from which nine hundred and sixty shoots were collected per cultivar at each sampling date. Ten to 15 centimeter long cuttings were then prepared from the current season's growth using the technique described by Couvillion and Erez (1980). This implied current stripping all the basal leaves off the cutting and leaving three to four terminal leaves on both terminal and basal cuttings. These cuttings were dipped for five seconds into an IBA solutions of 0, 500, 1000 and 2000 mg l^{-1} dissolved in 50:50 ethanol: water (v:v) or into a solution containing rooting-cofactor 100 µg l^{-1} added to the above mentioned

IBA solutions. A concentration of 100 μg l^{-1} rooting-cofactor was selected according to Hammerschlag *et al.* (1987).

After the five second dip into prepared rooting solutions the cuttings were allowed to dry at air temperature before being planted into the mistbed containing perlite. which was supplied with bottom heat (20 to 23°C) and an intermittent mist system to provide an environment conducive to cutting survival and development. Water was released onto the cuttings for 15 seconds at a six minute interval between sprays during summer months and a ten minute interval during winter months. Intermittent mist was produced by a solenoid valve in the water supply line. Treatments were tested using a randomized complete block design (RCBD) in a factorial arrangement consisting of two types of the cutting, three cultivars, four IBA concentrations and four rooting-cofactors (Appendix 1 and 2). Cuttings were inspected once a week to determine time and degree of rooting. After six weeks (normal rooting duration for *Prunus* species, Hartmann *et al.*, 1997) cuttings were removed from the mistbed and the percentage rooting and survival determined. The results were analyzed using GENSTAT 5 (4.1 Release, 4th edition, © Lawes Agricultural Trust IACR-Rothamsted).

2.2.2 Determination of the effect of IBA and Ca-EDTA on rooting of "wounded" and "non-wounded" peach hardwood cuttings

'Florida Prince' hardwood cuttings were prepared from one year old shoots. Ten cuttings of twenty centimeter long cuttings per treatment were sampled from three trees starting on 12 June 2000 to 28

July 2000 at bi-weekly interval. The treatments included IBA, wounding, no wounding and Ca-EDTA (Appendix 3).

The terminology of "wounding" referred to in the following describes an incision at the basal part of the cutting which was carried out in the following manner: The point of a budding knife was used to open shallow wounds on the cutting base. These shallow wounds were made in opposite sides of the cutting base penetrating to the outer cortex and extending upwards for about three cm. Cuts were made to a depth of one third stem thickness (Howard *et al.*, 1984). 'Wounded' and 'non-wounded' cuttings were dipped for five seconds to the depth of three cm into the respective solutions. IBA and IBA + Ca-EDTA solutions were made up as described in 2.2.1. Cuttings were then placed in the mist bed as described in After six weeks cuttings were removed from the bed and rooting assessed. The percentage of rooted cuttings, number of roots per cutting, average length of the roots and callus development (1=more callusing, 2=less callusing and 3= medium callusing) were recorded. Data were analyzed using GENSTAT 5 statistical package as previously described.

A concentration of 1000 mg l^{-1} IBA had been found to be optimal for rooting of softwood peach cuttings (2.3.1),this concentration was used to evaluate the effect of Ca-EDTA on rooting. Calcium was applied as a chelate because it does not penetrate membranes with ease as Ca^{++}. Calcium was evaluated for its effect on rooting of peach cuttings as it has been found to have a marked influence on rooting (Bellamine *et al.*, 1998). A complete randomized design (CRD) was used consisting of eight treatments, three replications and ten cuttings per treatment.

2.2.3 Determination of sugars in softwood cuttings of peach cv Florida Prince during the ARF process

2.2.3.1 Plant material

Shoots of 'Florida Prince' were collected from four trees. A total of 240 shoots was sampled in October an ten cuttings were used per treatment. The bases of softwood 'Florida Prince' peach cuttings were either dipped into distilled water (control) or into a 1000 mg l^{-1} of IBA solution and rooted under rooting conditions described previously (chapter 2.2.1). The cuttings were sampled at zero, two, four and six weeks after being placed into a mist bed as described in 2.1.1. Three mm of the cutting bases were cut off and freeze-dried immediately after cuttings were removed from the mistbed. After freeze-drying the plant material was ground to a powder with a molar and pestle and five grams (dry weight) of each sample was stored in a deep freeze at -18°C for carbohydrate analysis.

2.2.3.2 Starch quantification

Starch analysis was carried out according to Buysse and Merckx (1993). A sample of 0.05 g (dw) was extracted in 5 ml of 80 % (v/v) ethanol:water and centrifuged at 152 G for 20 minutes in a Hermle Z510 centrifuge (Zeiss, Germany (Pty) Ltd). The supernatant was discarded and the pellet dissolved in 5 ml of 80 % (v/v) ethanol:water. This process was repeated twice before the pellet was dissolved in ultra-pure water and hydrolyzed for three hours in boiling concentrated hydrochloric acid. An aliquot of 400 µl of each sample was pipetted into a test tube, to which 400 µl phenol solution (Merck, Darmstadt, Germany) and 5 ml concentrated sulphuric acid were added. The samples were mixed by a vortex shaker and allowed to stand for at least 15 minutes before the

absorbance was read at 490 nm using a s spectrophotometer (Anthelic Cedex, Secomom Co., France). Total carbohydrates were determined by comparison to D-glucose from the above supernatant. A standard curve was prepared with 100, 80, 60, 40, 20, and 0μg.ml^{-1} D-glucose (Sigma, St. Louis, USA) (Appendix 4).

2.2.3.3 Quantification of sugars

The sample preparation for determination of soluble carbohydrates was carried out according to Buysse and Merckx (1993) and was similar to the one carried out for starch analysis. The ground samples were however, not hydrolyzed in 3 % concentrated hydrochloric acid and only the precipitate dissolved in five ml 80 % (v/v) ethanol was used. The sample was filtered through milipore filters (0.45 μm) and 100μl of the filtered extract was injected into an HPLC system (Model ERC-7515A, ERC INC., Tokyo, Japan), using the following specifications: Column: Rezex 8μm 8 % Ca Monos: 300×7.80 mm, mobile phase: water (ultra pure); flow rate: 0.5ml/min; temperature: 75°C; injection volume: 100μl; detector: refractive index at 40°C. Sugar quantification was achieved by comparing sample peaks at the retention times of previously prepared standards of sucrose, fructose, glucose, sorbitol and mannitol according to Richings *et al.* (2000).

2.3 Results

2.3.1 Effect of IBA, rooting-cofactors and season on the rooting of terminal and basal stem cuttings of three peach cultivars

Spring and early summer cuttings yielded significantly higher rooting percentage in all three cultivars tested. Softwood cuttings displayed a higher rooting percentage than hardwood cuttings,

particularly with the lower IBA used (500 mg l^{-1}) (Fig. 2.1 B, 2.2 B and 2.3 B). Differences were furthermore observed between the two cutting positions, with the terminal position obtaining highest rooting percentages mostly in spring and early summer at lower IBA concentrations (Fig. 2.1 and Fig 2.3). Autumn and winter basal cuttings had higher rooting percentages compared to terminal cuttings (Fig 2.1 to Fig 2.3). 'Florida Prince' displayed the overall highest rooting percentage (74 %) (Fig 2.2, A to D), followed by 'DeWet' (62 %) (Fig 2.1, A to D) and 'Earlibelle' (54 %) (Fig. 2.3 A to D).

The effect of rooting-cofactors varied for different seasons (Fig. 2.4 to Fig. 2.15). Phloroglucinol (Fig 2.4, 2.8 and 2.12) and chlorogenic acid (Fig 2.5, 2.9 and 2.13) influenced the rooting percentage positively. Quercitin (Fig 2.6, 2.10 and 2.14) and rutinin (Fig 2.7, 2.11 and 2.15), on the other hand did not show a significant influence on rooting of the cultivars studied throughout the four seasons, although higher rooting percentages were obtained when compared to IBA treatments.

The season of cutting preparation proved to important factor in rooting peach cuttings. Overall, cuttings rooted best and responded best to the applied treatment in October (spring) and November (early summer) (Fig. A and B of 2.4 to 2.15), a time corresponding to the developmental stage in growth and production of new succulent shoots.

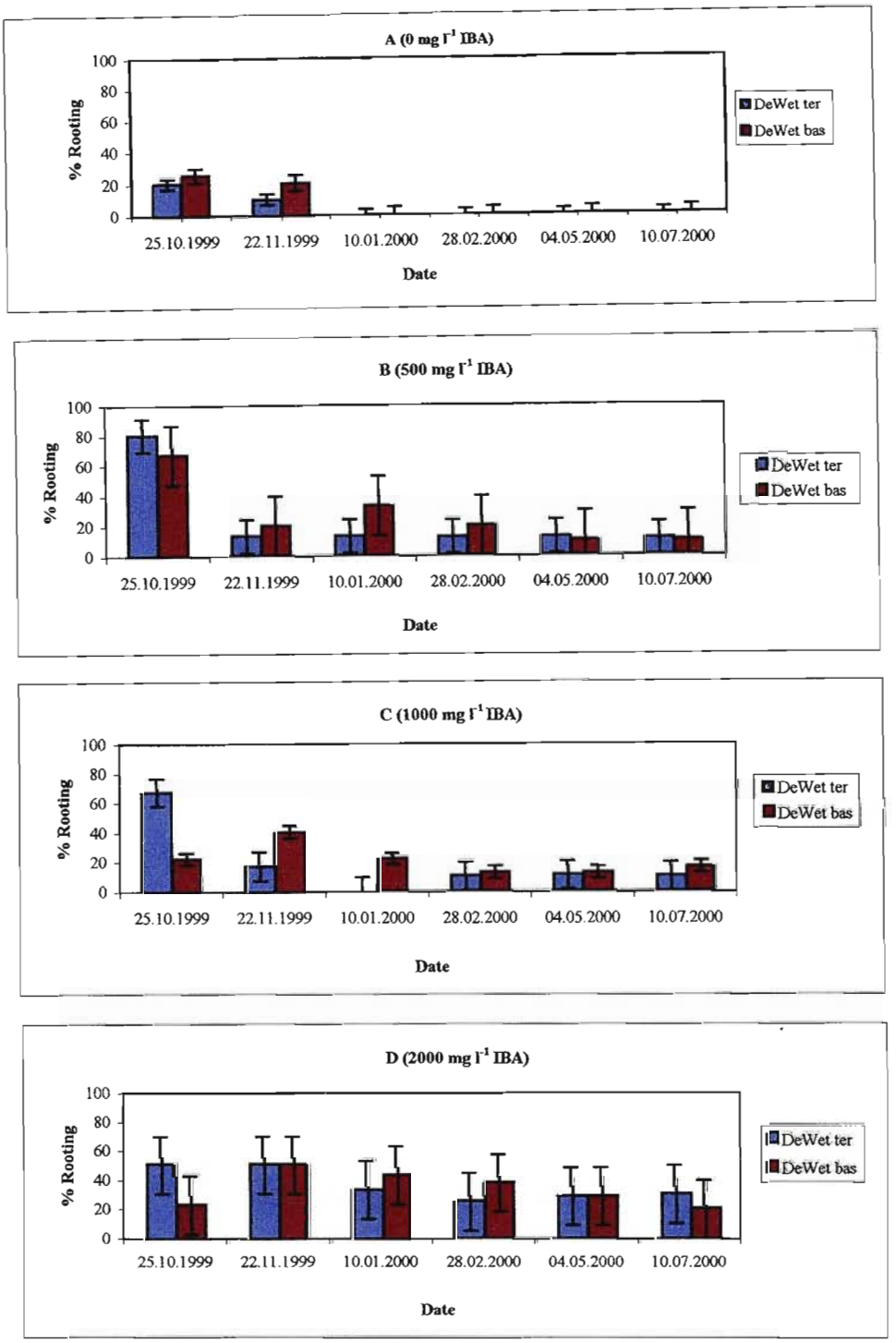


Fig 2.1: Influence of different concentrations of IBA on rooting of cv. DeWet (A, B, C and D) during spring, summer, autumn and winter.

Cuttings were evaluated for rooting after six weeks in the mist bed. Data are means of three replications, each treatment consists of 10 cuttings. Fitted bar charts are standard type columns (clustered columns) with standard errors at P=0.05, created by the statistical analysis function of Microsoft Excel.

Abbreviations: DeWet ter ('DeWet' termianl cutting position) and DeWet bas ('DeWet' basal cutting position)

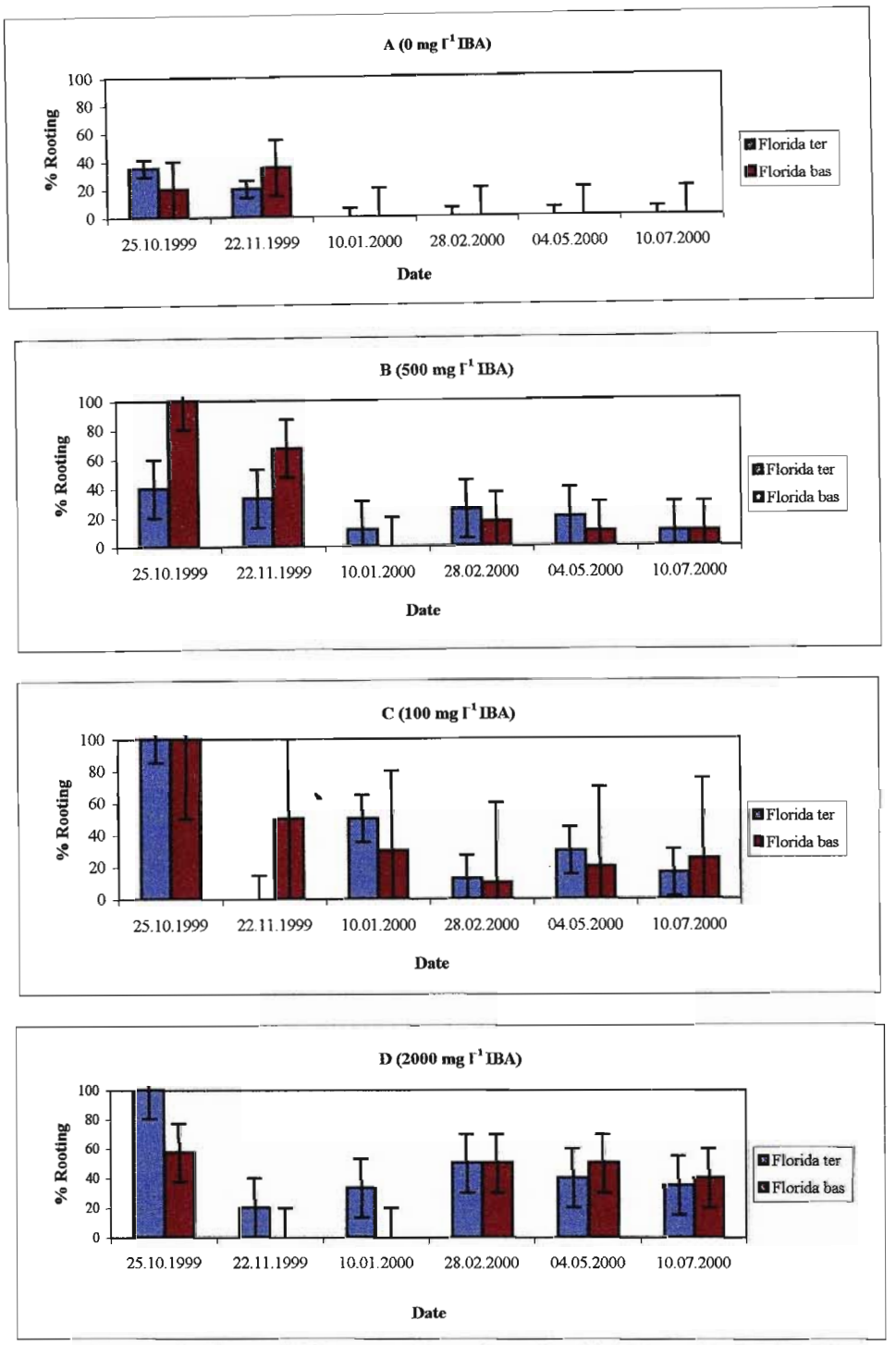


Fig 2.2: Influence of different concentrations of IBA on rooting of cv. Florida Prince (A, B, C and D) during spring, summer, autumn and winter.

Cuttings were evaluated for rooting after six weeks in the mist bed. Data are means of three replications, each treatment consists of 10 cuttings. Fitted bar charts are standard type columns (clustered columns) with standard errors at P=0.05, created by the statistical analysis function of Microsoft Excel.

Abbreviations: Florida ter ('Florida Prince' terminal cutting position) and Florida bas ('Florida Prince' basal cutting position)

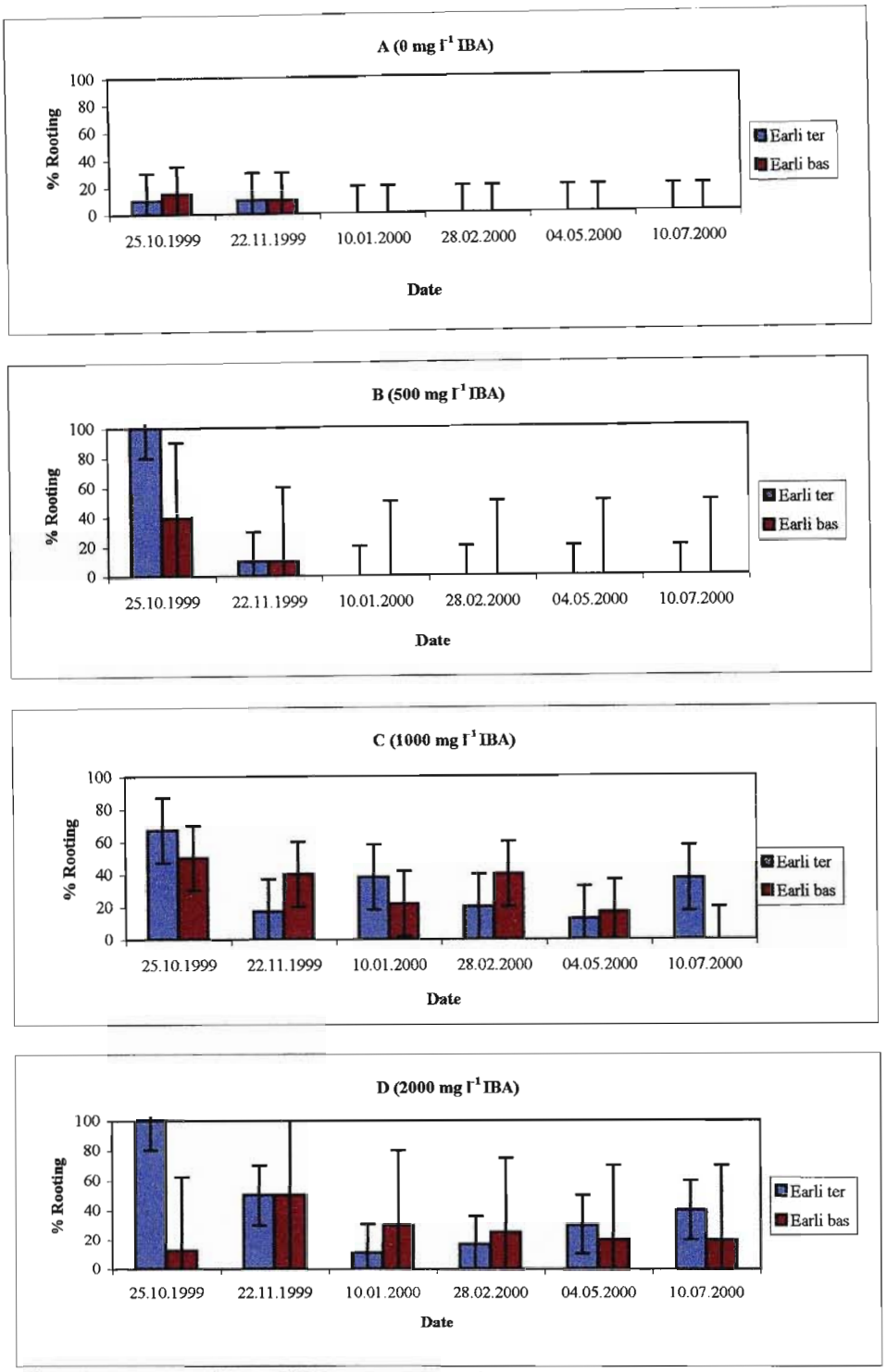


Fig 2.3: Influence of different concentrations of IBA on rooting of cv.Earlibelle (A, B, C and D) during spring, summer, autumn and winter.

Cuttings were evaluated for rooting after six weeks in the mist bed. Data are means of three replications, each treatment consists of 10 cuttings. Fitted bar charts are standard type columns (clustered columns) with standard errors at P=0.05, created by the statistical analysis function of Microsoft Excel.

Abbreviations: Earli ter ('Earlibelle' terminal cutting position) and Earli bas Earlibelle' basal cutting position)

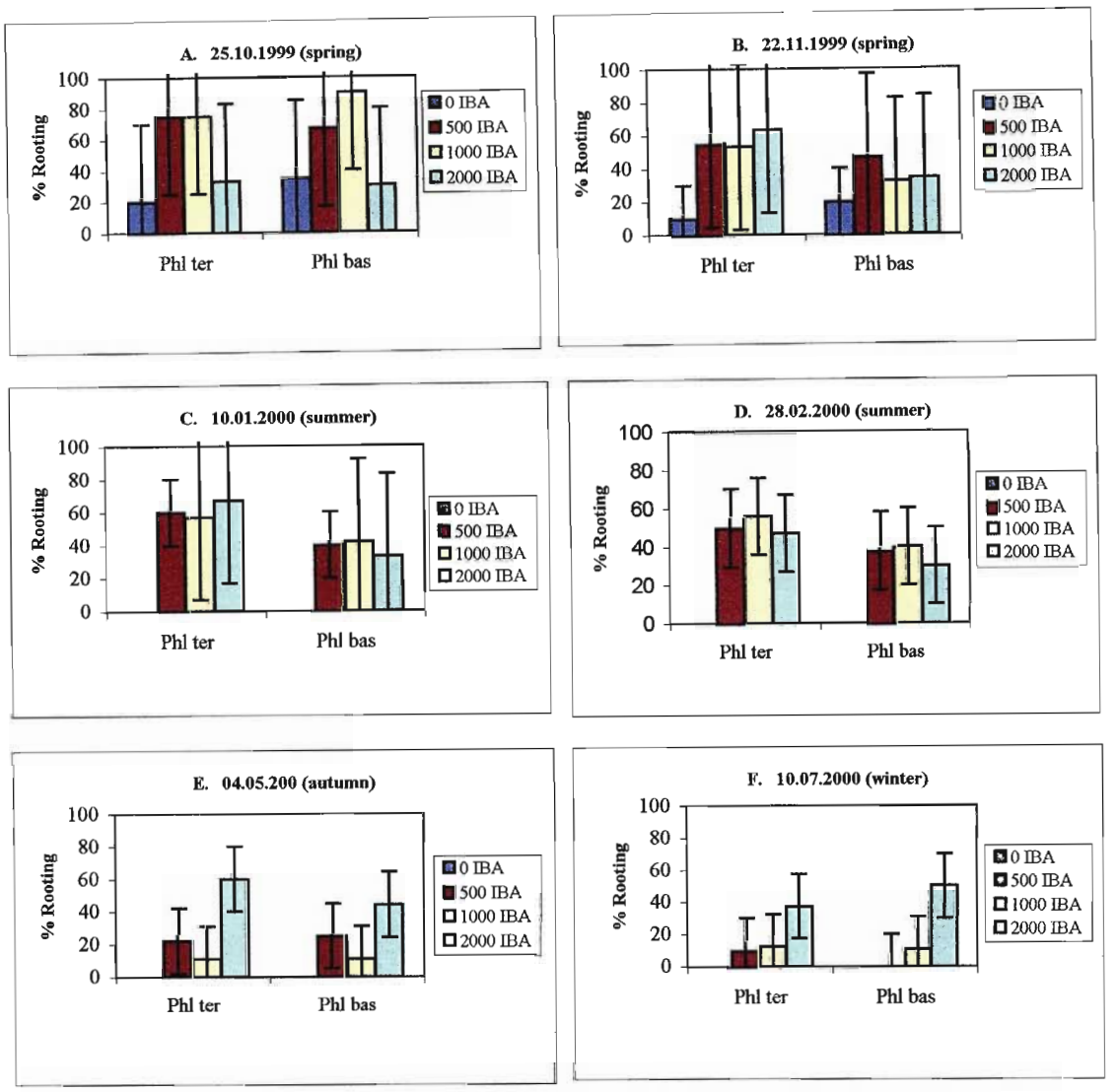


Fig 2.4: Effect of phloroglucinol and IBA concentrations on rooting of 'DeWet' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and phloroglucinol (100µg l^{-1}) Data were collected six weeks after the cuttings were planted in the mistbed for rooting. Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.

Abbreviations: Phl (Phloroglucinol), ter (terminal cutting position) and bas (basal cutting position)

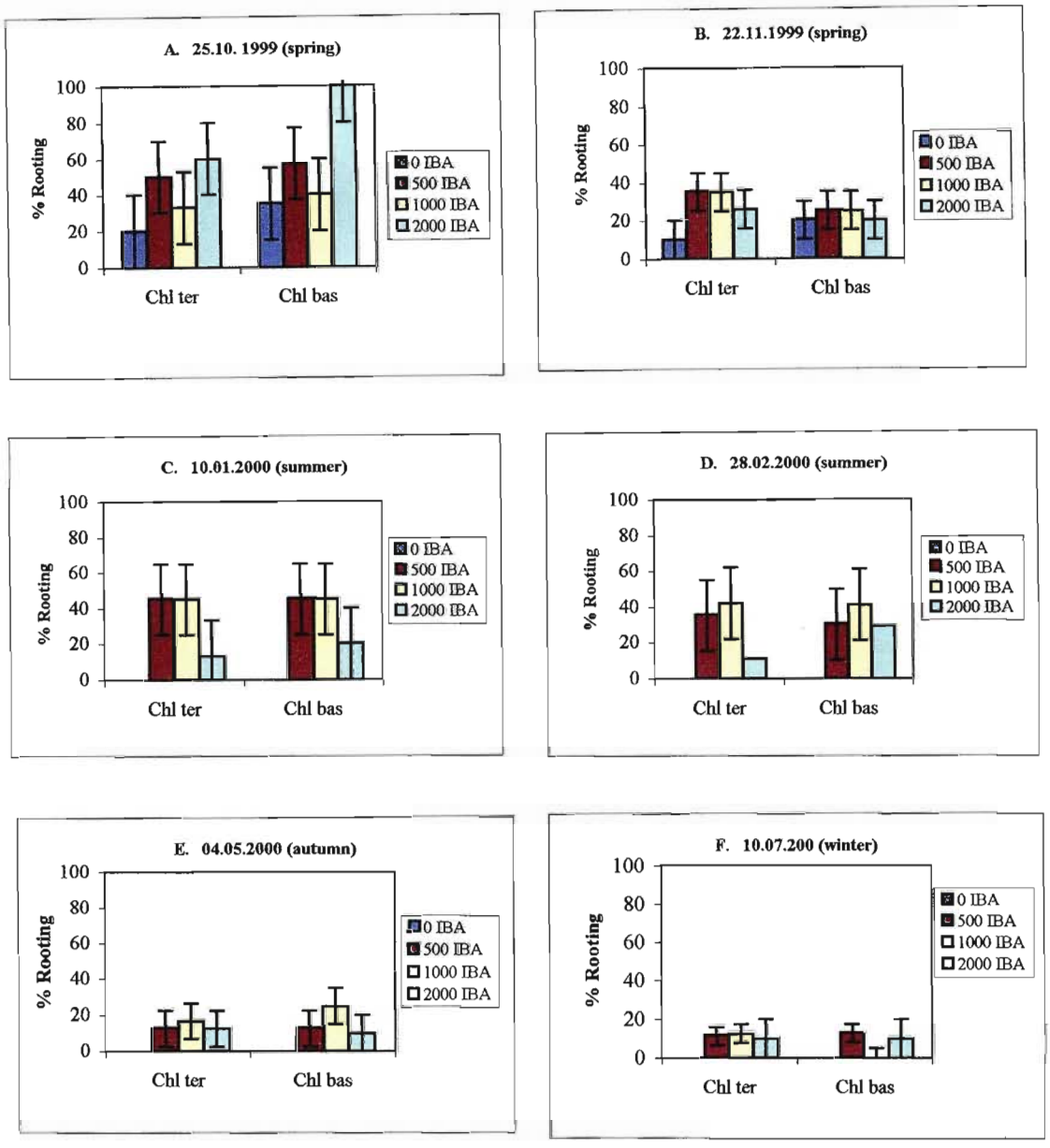


Fig 2.5: Effect of chlorogenic acid and IBA concentrations on rooting of 'DeWet' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and chlrogenic acid (100µg l^{-1}) Data were collected six weeks after the cuttings were plated in the mistbed for rooting. Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05, created by the statistical analysis function of Microsoft Excel.

Abbreviations: Chl (chlorogenic acid), ter (terminal cutting position) and bas (basal cutting position)

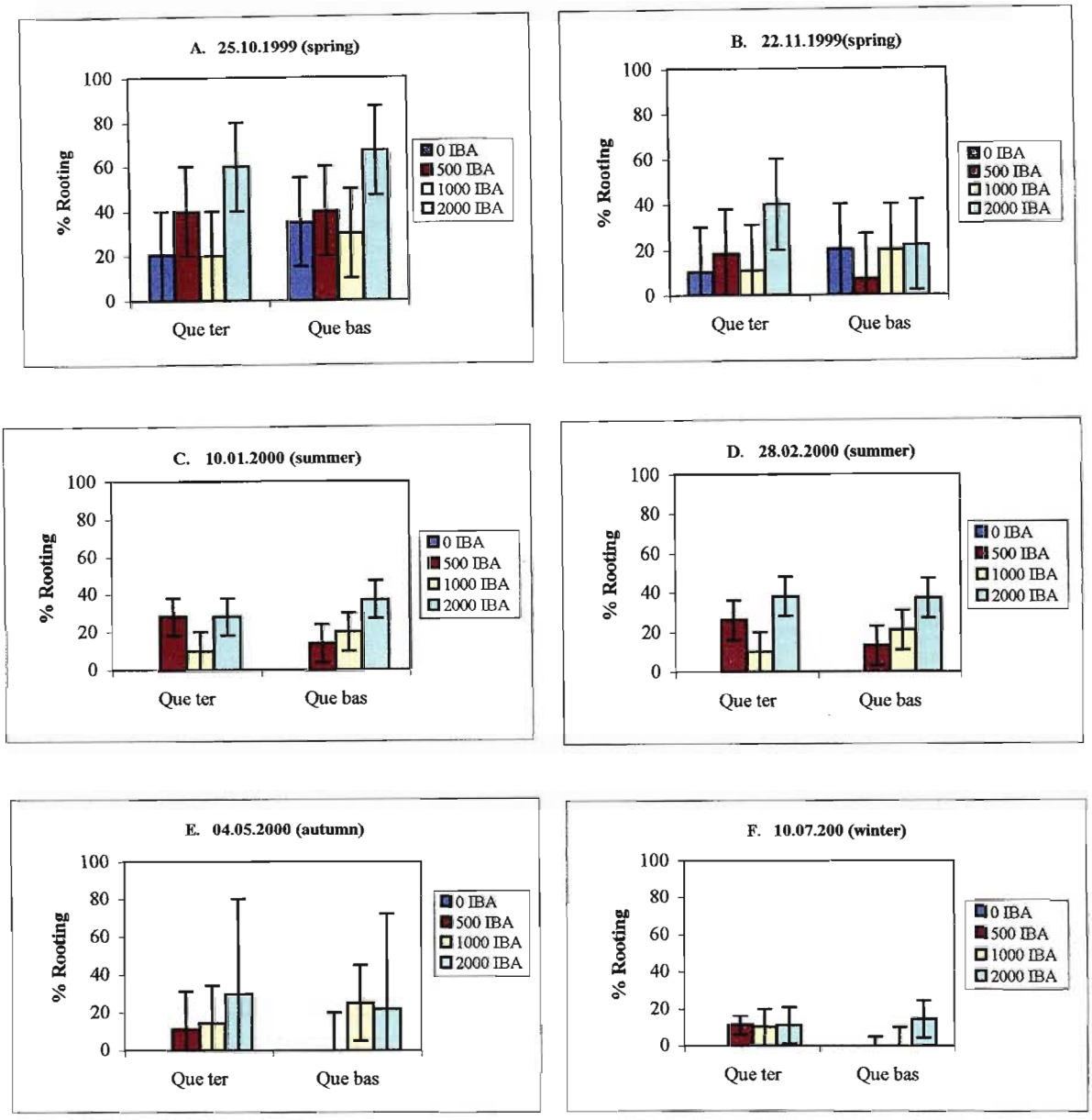


Fig 2.6: Effect of quercitin and IBA concentrations on rooting of 'DeWet' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and quercitin (100µg l^{-1})
Data were collected six weeks after the cuttings were planted in the mistbed for rooting.
Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.
Abbreviations: Que (Quercitin), ter (terminal cutting position) and bas (basal cutting position)

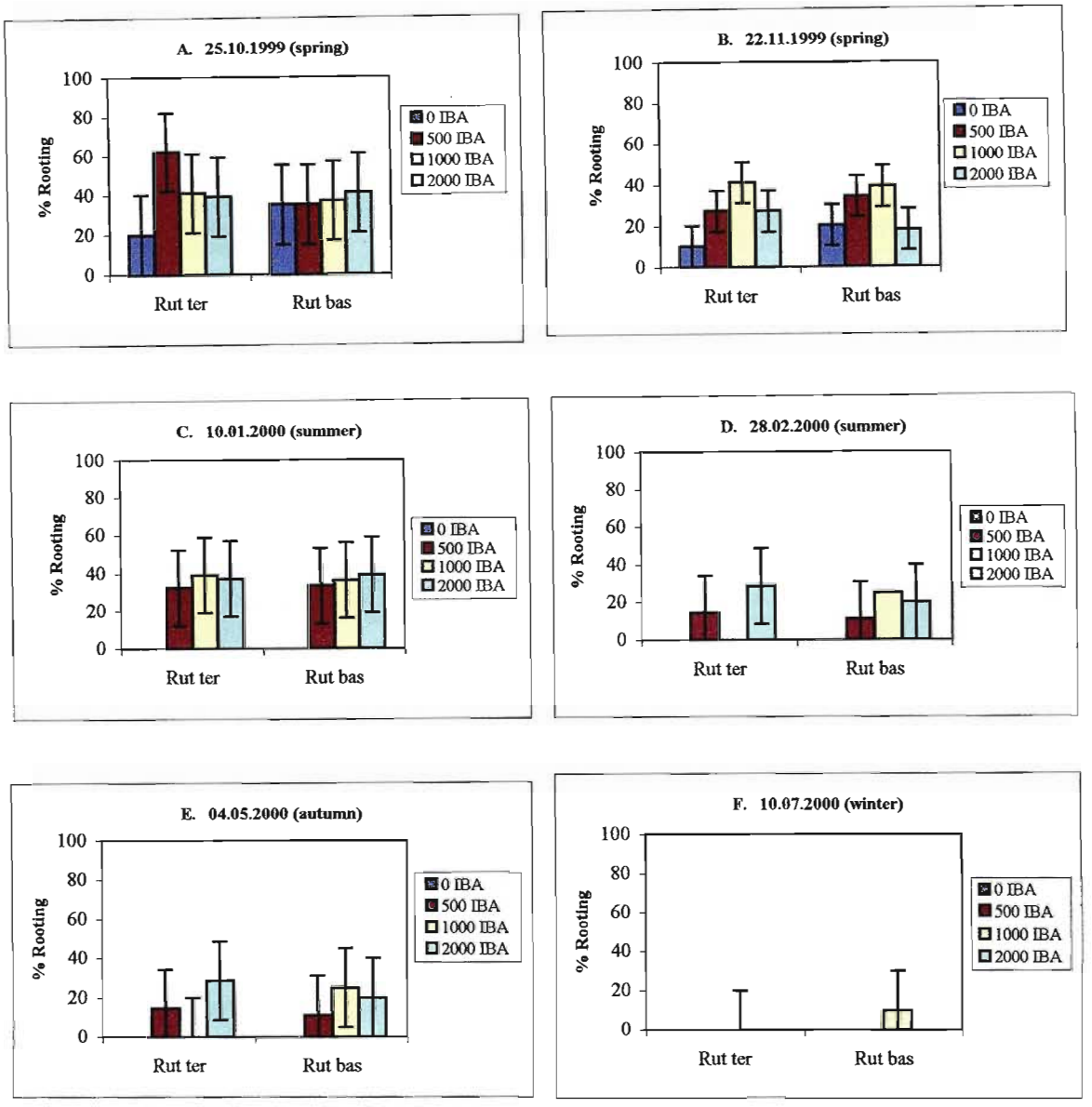


Fig 2.7: Effect of rutinin and IBA concentrations on rooting of 'DeWet' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and rutinin (100µg l^{-1})
Data were collected six weeks after the cuttings were planted in the mistbed for rooting.
Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.
Abbreviations: Rut (Rutinin), ter (terminal cutting position) and bas (basal cutting position)

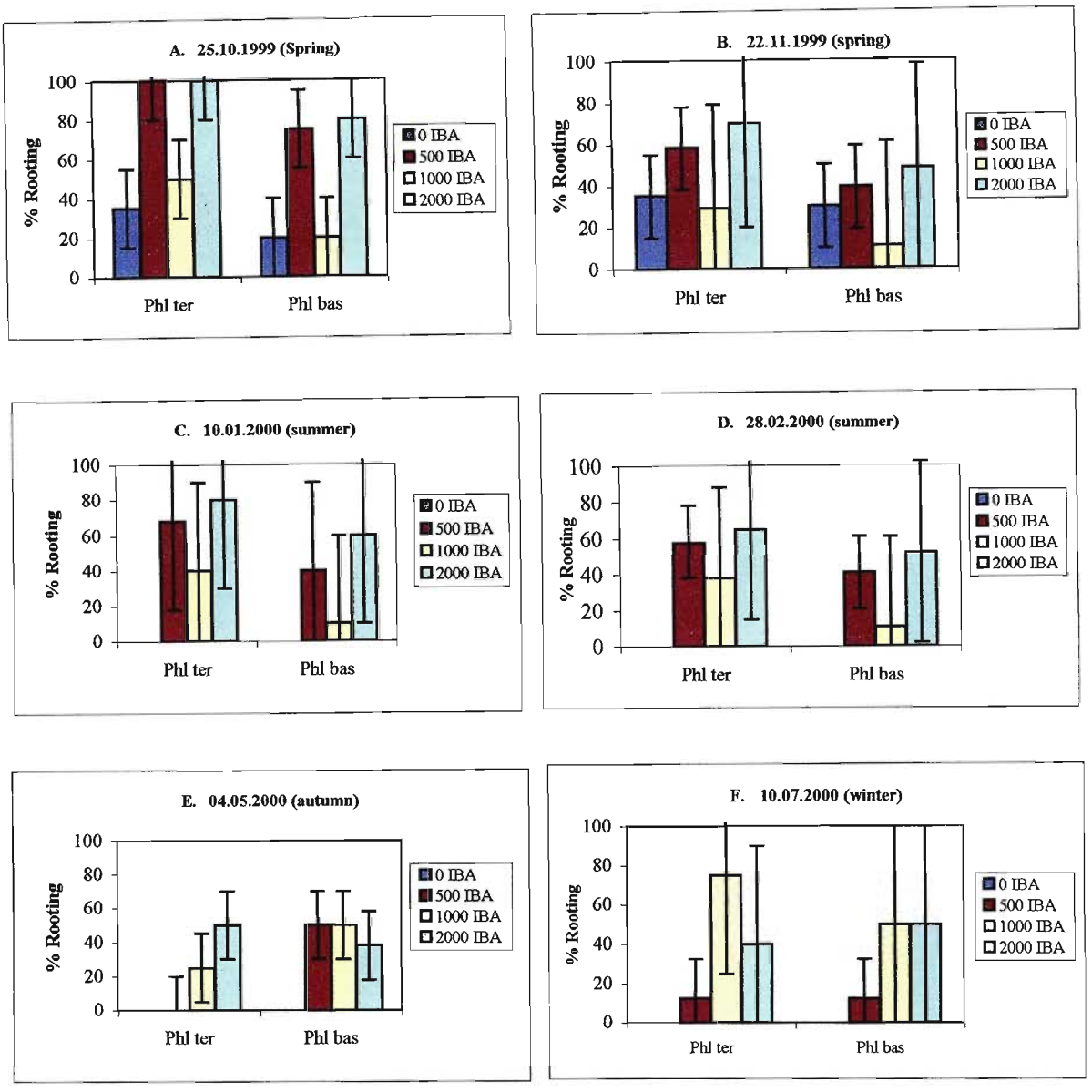


Fig 2.8: Effect of phloroglucinol and IBA concentrations on rooting of 'Florida Prince' cuttings at different sesons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and phloroglucinol (100µg l^{-1}) Data were collected six weeks after the cuttings were planted in the mistbed for rooting. Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.

Abbreviations: Phl (Phloroglucinol), ter (terminal cutting position) and bas (basal cutting position)

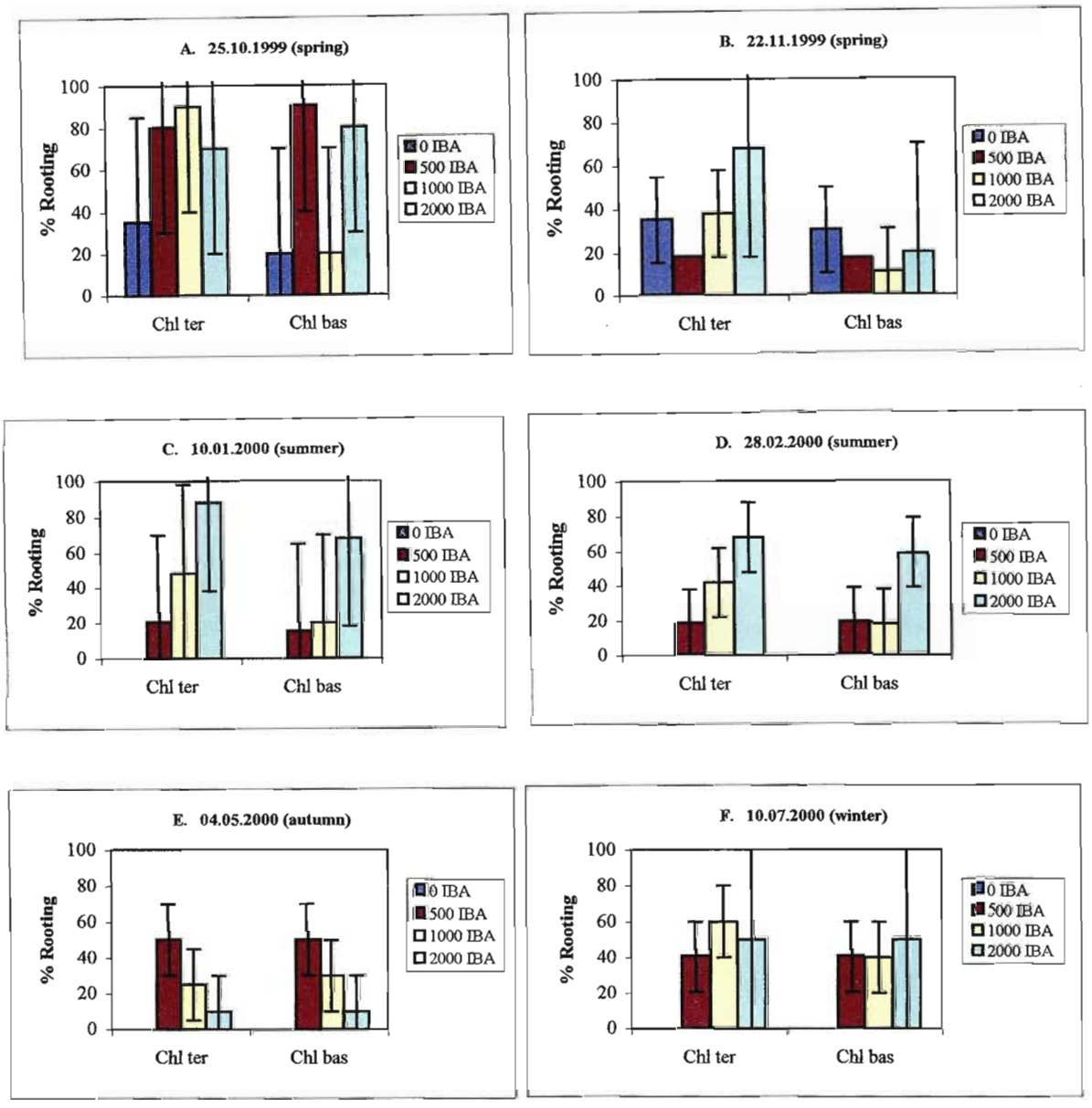


Fig 2.9: Effect of chlorogenic acid and IBA concentrations on rooting of 'Florida Prince' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and chlorogenic acid (100µg l^{-1}) Data were collected six weeks after the cuttings were planted in the mistbed for rooting. Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.

Abbreviations: Chl (Chlorogenic acid), ter (terminal cutting position) and bas (basal cutting position)

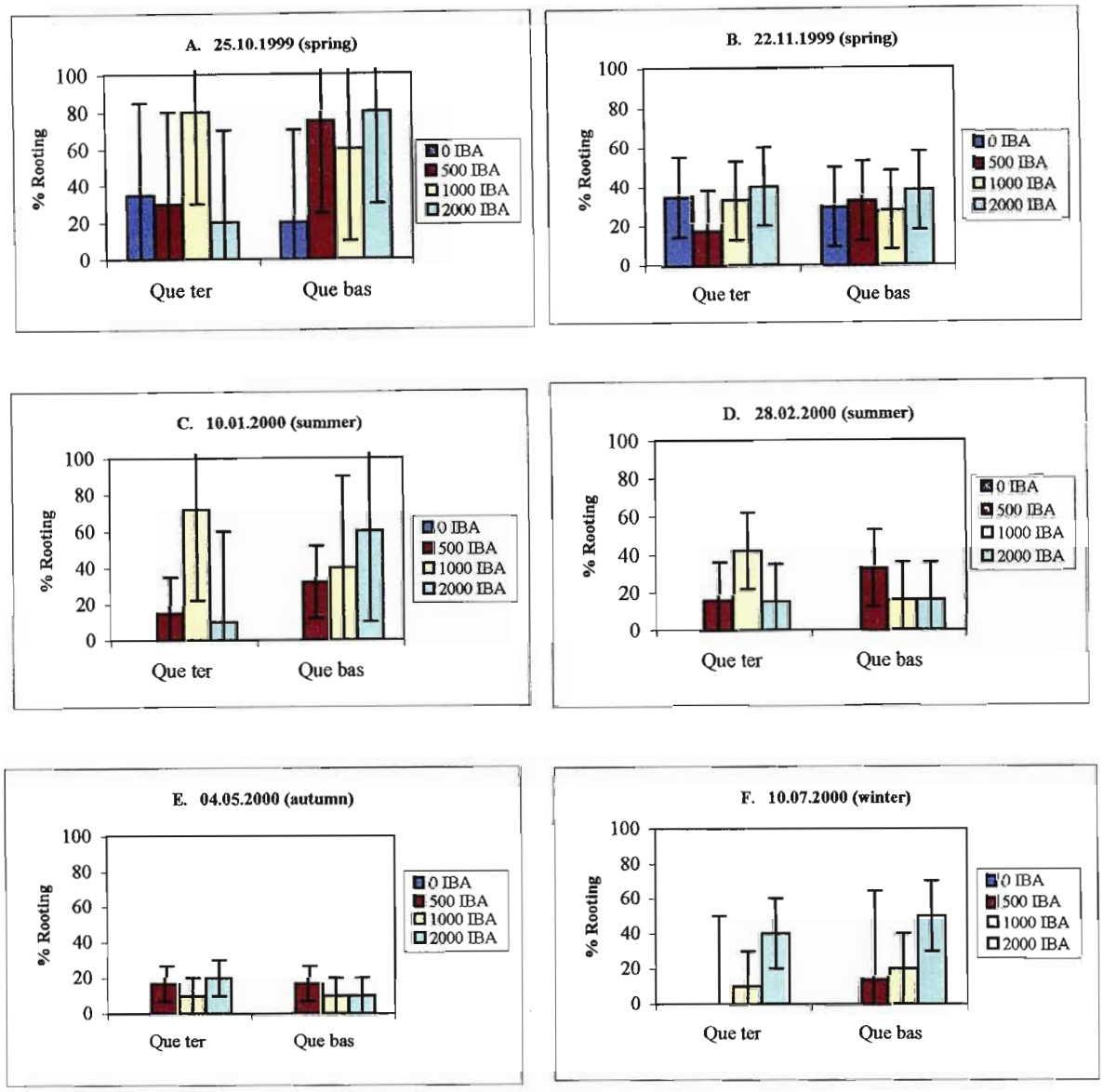


Fig 2.10: Effect of quercitin and IBA concentrations on rooting of 'Florida Prince' cuttings at different sesons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and quercitin (100µg l^{-1})
Data were collected six weeks after the cuttings were planted in the mistbed for rooting.
Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.
Abbreviations: Que (Quercitin), ter (terminal cutting position) and bas (basal cutting position)

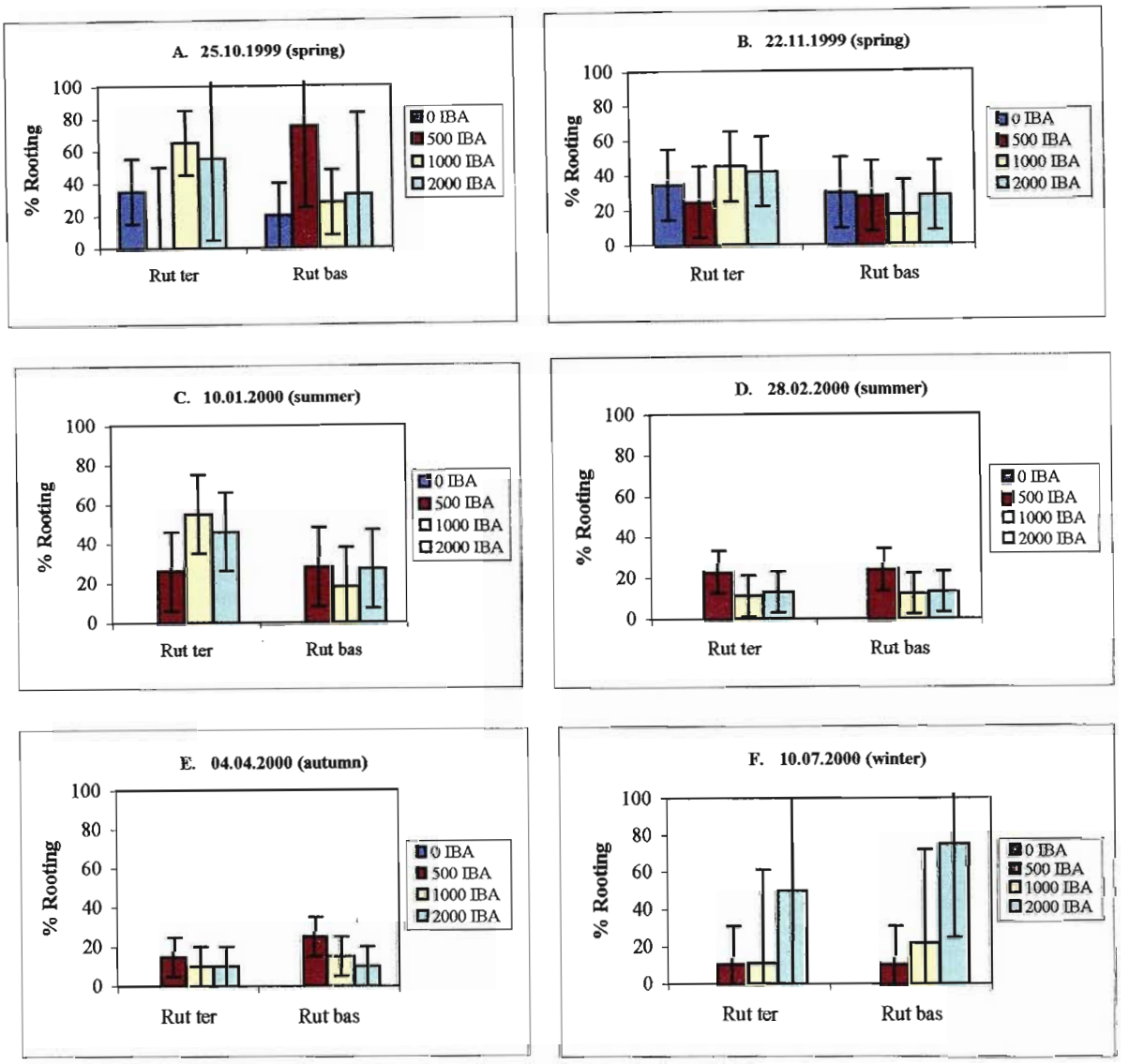


Fig 2.11: Effect of rutinin and IBA concentrations on rooting of 'Florida Prince' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and quercitin (100µg l^{-1})
Data were collected six weeks after the cuttings were planted in the mistbed for rooting.
Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.
Abbreviations: Rut (Rutinin), ter (terminal cutting position) and bas (basal cutting position)

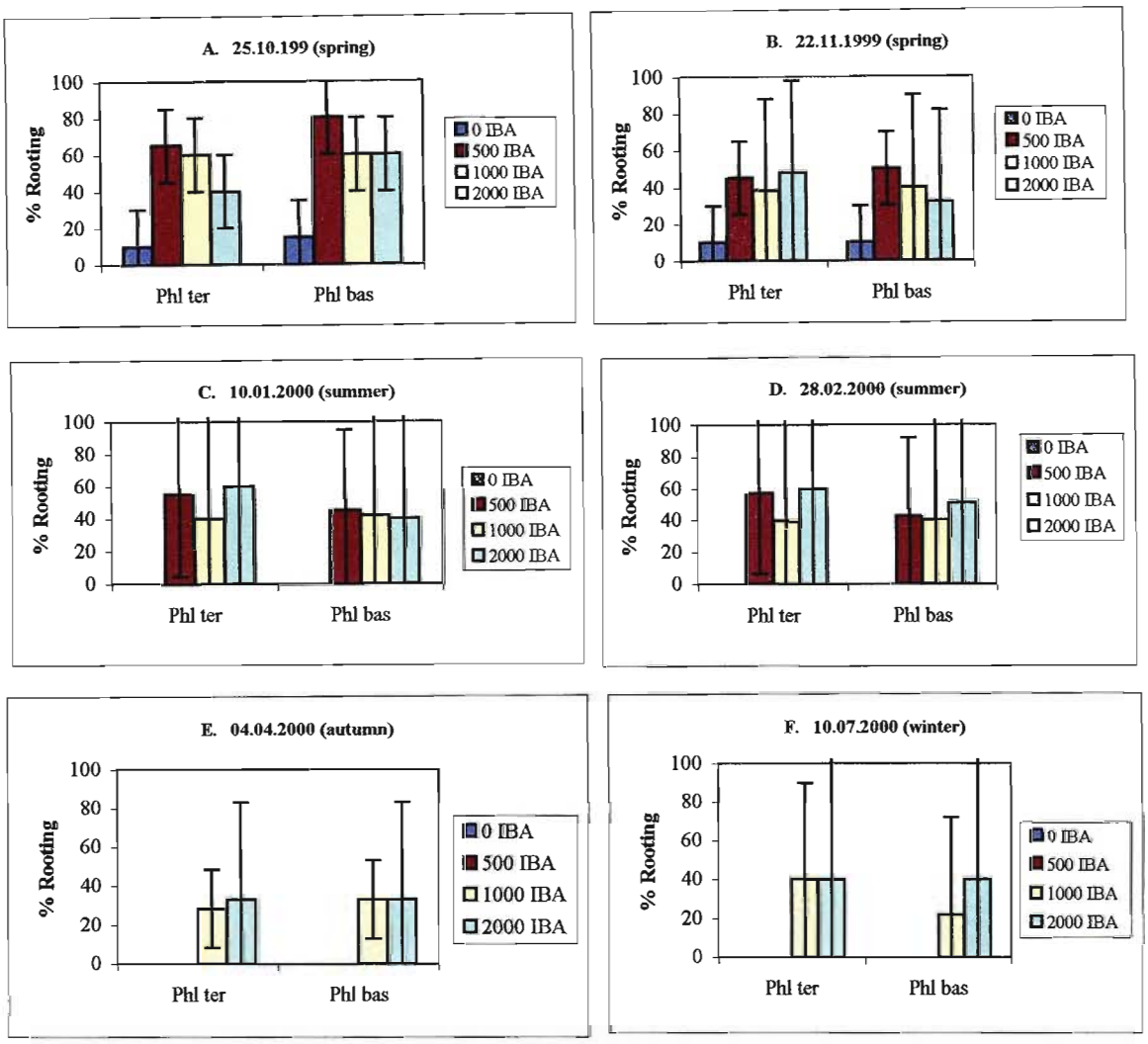


Fig 2.12: Effect of phloroglucinol and IBA concentrations on rooting of 'Earlibelle' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and quercitin (100µg l^{-1})
Data were collected six weeks after the cuttings were planted in the mistbed for rooting.
Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.
Abbreviations: Phl (Phloroglucinol), ter (terminal cutting position) and bas (basal cutting position)

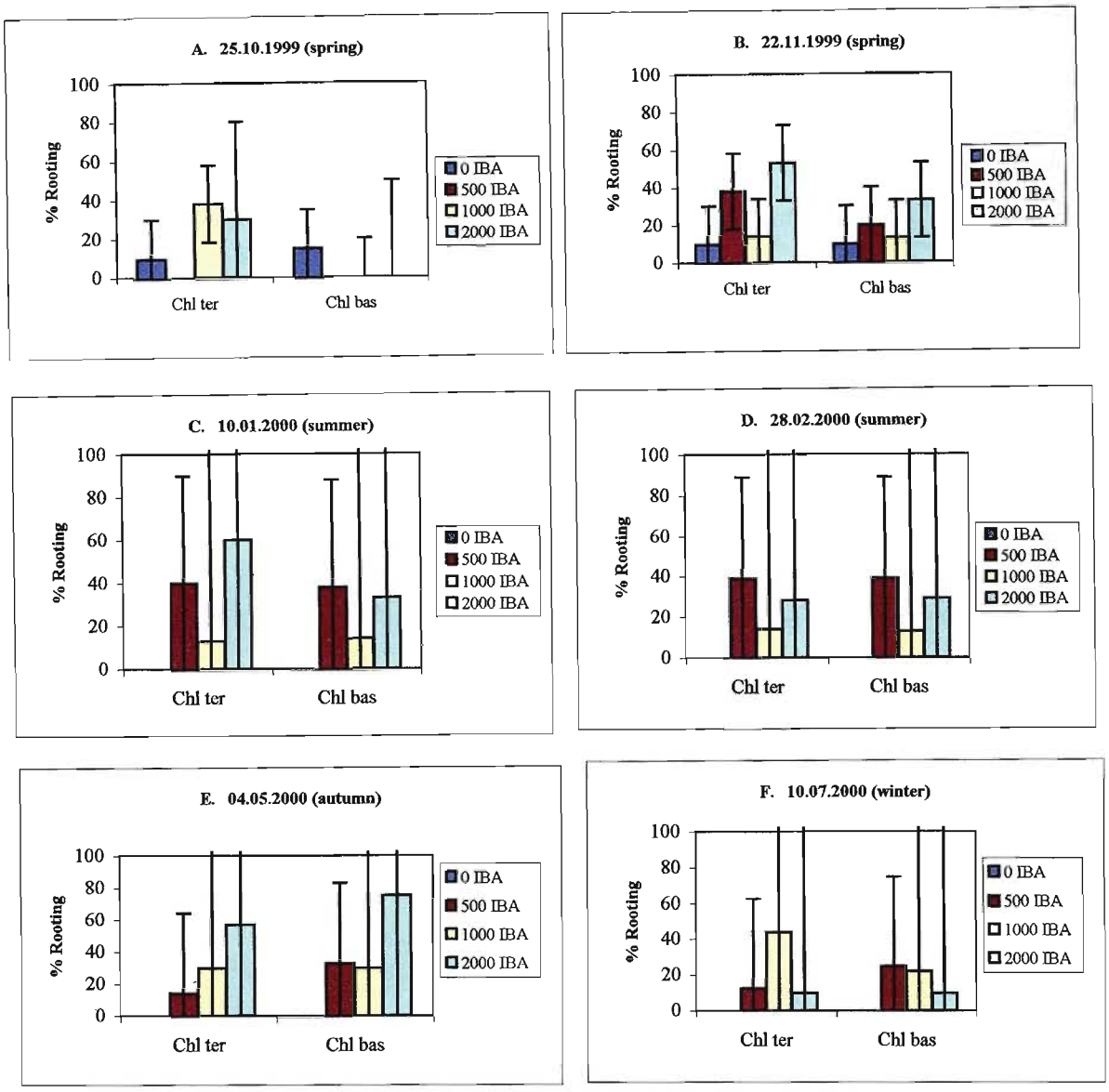


Fig 2.13: Effect of chlorogenic acid and IBA concentrations on rooting of 'Earlibelle' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and quercitin (100µg l^{-1})
Data were collected six weeks after the cuttings were planted in the mistbed for rooting.
Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.
Abbreviations: Chl (Chlorogenic acid), ter (terminal cutting position) and bas (basal cutting position)

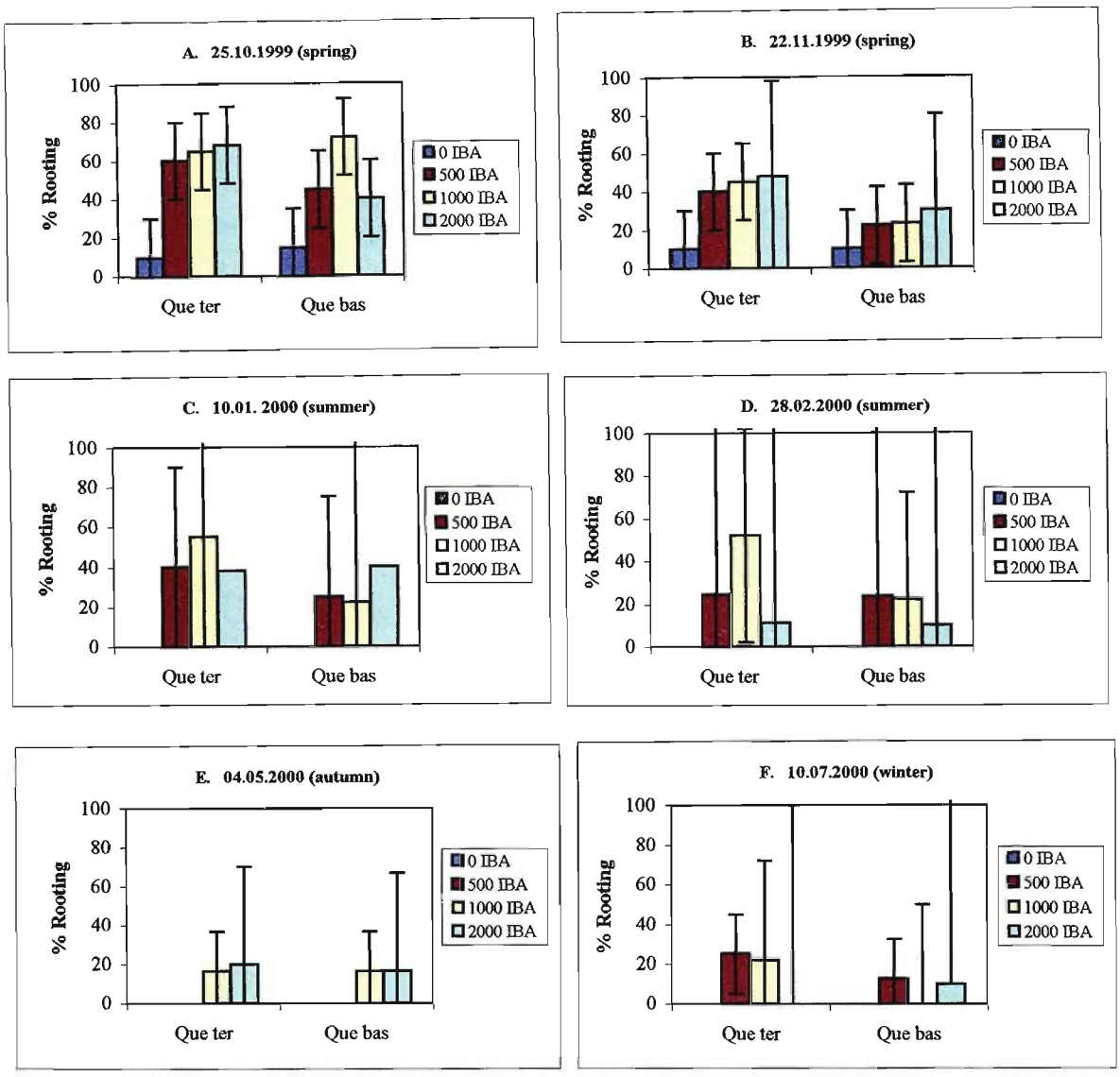


Fig 2.14: Effect of quercitin and IBA concentrations on rooting of 'Earlibelle' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and quercitin (100µg l^{-1})
Data were collected six weeks after the cuttings were planted in the mistbed for rooting.
Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05, created by the statistical analysis function of Microsoft Excel.
Abbreviations: Que (Quercitin), ter (terminal cutting position) and bas (basal cutting position)

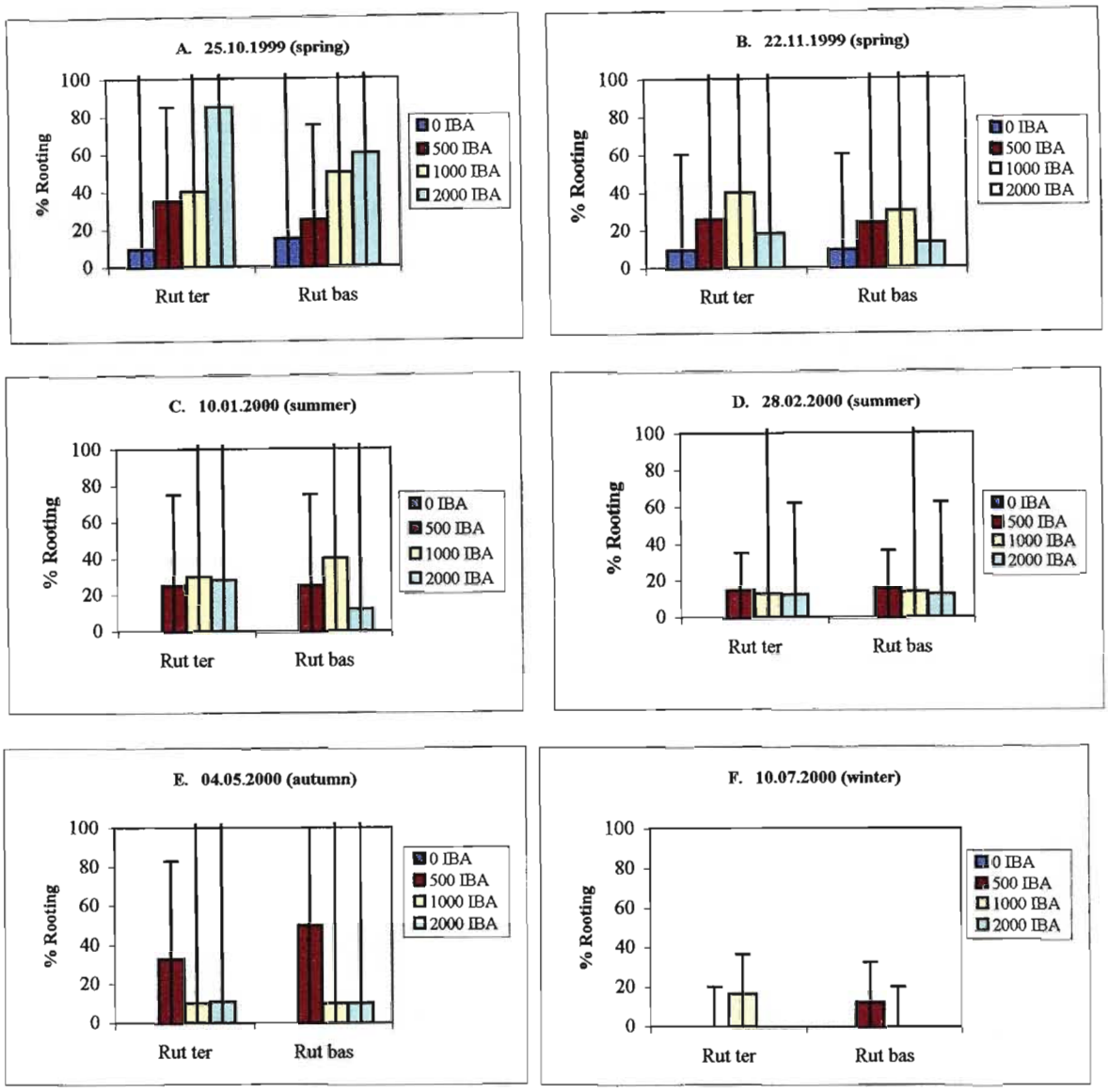


Fig 2.15: Effect of rutinin and IBA concentrations on rooting of 'Earlibelle' cuttings at different seasons (A, B, C, D, E and F)

Cuttings were dipped in a solution containing IBA (mg l^{-1}) and quercitin (100µg l^{-1})
Data were collected six weeks after the cuttings were planted in the mistbed for rooting.
Each treatment consists of 10 cuttings replicated three times. Fitted bar charts are standard type columns (clustered column), with standard errors at P=0.05 created by the statistical analysis function of Microsoft Excel.
Abbreviations: Rut (Rutinin), ter (terminal cutting position) and bas (basal cutting position)

2.3.2 Effect of IBA and Ca-EDTA on rooting of "wounded" and "non-wounded" peach hardwood cuttings

The combination of IBA (1000 mg l^{-1}) with "wounding" (basal splitting) and Ca-EDTA (100 mg l^{-1}) gave the highest rooting percentage (86.7 %; P=0.05), longest root per cutting (11.70 cm; P=0.05) and highest number of roots per cutting (13.33; P=0.05) while 0 mg l^{-1} IBA and "no wounding" resulted in lower rooting percentage after rooting period of six weeks. It was observed, however, that callus production in response to the above treatment ("wounded tissues") intensifies more along the longitudinal axis of the wound/incision than at the base of the cutting. The cuttings that were wounded but did not receive any application of IBA or Ca-EDTA gave significantly poorer results when compared to the control (P=0.05). There were very small but significant, differences observed between various treatments (Table 2.1). The analysis of variance (Appendix 6) indicated that all IBA and Ca-EDTA treatments were significantly different compared to the control ($P \leq 0.01$). The combination of Ca-EDTA and IBA increased the ability of cuttings to root. The rooting percentage was however, not affected by application of Ca- EDTA (Table 2.1), but the IBA application increased rooting percentage compared to Ca-EDTA (Table 2.1). The combination of Ca-EDTA, IBA and wounding gave the highest rooting percentage of 'Florida Prince' hardwood cuttings (P=0.05). "Wounding" alone did not affect the rooting percentage, however, combination of Ca-EDTA or IBA with "wounding" resulted in an increased rooting percentage.

Table 2.1 : Effect of "wounding", IBA and Ca-EDTA on rooting of 'Florida Prince' peach hardwood cuttings

Treatments	% Rooting	Average number of roots/cutting	Average length of roots/cutting (cm)
CONTROL	0.00	0.00	0.00
1000 mg l^{-1} IBA	33.3	8.67	6.40
Ca-EDTA (100 mg l^{-1})	10.0	2.00	5.30
Wounding	6.7	4.33	1.93
1000 mg l^{-1} IBA + Wounding	73.3	8.7	10.0
1000 mg l^{-1} IBA + Ca-EDTA (100 mg l^{-1})	33.3	3.7	4.9
Ca-EDTA (100 mg l^{-1}) + Wounding	23.3	6.0	4.2
1000 mg l^{-1} IBA + Ca-EDTA (100mg l^{-1}) + Wounding	86.7	13.3	11.7
LSD (1 %)	22.1	4.8	3.8
± SEM	10.4	2.9	1.8
CV (%)	38	48	39.2

Each treatment consisted of ten cuttings and was replicated three times. Experiments were arranged in a complete randomized design (CRD). Cuttings were dipped in a solution containing IBA and Ca-EDTA and planted in the mistbed for six weeks. Data were analyzed by Genstat 5, 4.1 Release, 4th Edition, Lawes Agricultural Trust, IARC, Rothamsted.

Abbreviations: Ca-EDTA (Calcium-ethylene-diamine tetra acetic acid), IBA (Indolebutyric acid), LSD (1 %) (Least significant difference at 99 % confidence), CV (coefficient of variation) and ± SEM (standard error of the mean)

2.3.3 Role of sugars in peach softwood cuttings during the adventitious rooting process

Sucrose, fructose and glucose were found to be higher at first sampling date (4th October 2000) compared to the following sampling dates in both control and IBA-treated cuttings (Table 2.2). The

concentration of sucrose, glucose and fructose decreased from the first date to the second sampling date and remained at a low level thereafter .The sugar alcohols (mannitol and sorbiltol) followed a different pattern, there were low concentrations during first sampling dates followed by slight accumulations during subsequent dates. Higher sugar levels (except sugar alcohols) were obtained in tissues treated with IBA as opposed to the treated cuttings (Table 2.2). However, the relative proportions of sugars either decreased or remained constant over the sampling period (Table 2.2). The level of sucrose decreased with subsequent sampling dates , but there was no statistical difference between different sampling dates. A similar trend was also followed in cuttings treated with 1000 mg l^{-1} IBA (Table 2.2). There was no significant decrease in sugars from preparation of cuttings to 42 days in the mistbed. Glucose was the only carbohydrate measured which changed while the cuttings were in the mistbed. However, the glucose levels increased towards final stages of rooting (five to six weeks after planting the cuttings in the mistbed), to a higher concentration of 10.0 mg g^{-1} dw, compared to the other two sugars which reached only 3 mg g^{-1} dw concentrations. Sorbitol and mannitol concentrations fluctuated throughout the entire rooting process .

Table 2.2: Soluble sugar, sugar alcohols and starch levels in peach 'Florida Prince' basal softwood cuttings during the ARF process.

Treatment mg l^{-1} IBA	Time in mistbed (days)	Sucrose mg g^{-1}dw	Fructose mg g^{-1}dw	Glucose mg g^{-1}dw	Sorbitol mg g^{-1}dw	Mannitol mg g^{-1}dw	Soluble sugar mg g^{-1}dw	Starch mg g^{-1}dw
0	0	7.0a	8.0a	7.0a	4.9a	1.5a	0.2a	0.3a
	14	3.5b	1.4b	1.1b	13.2a	4.6a	0.5a	0.2a
	28	2.3b	1.4b	0.9b	4.0a	26.8a	0.2a	0.2a
	42	0b	1b	2.9c	4.9a	5.8a	0.2a	0.2a
1000								
	0	14	3.5a	1.6b	1.1c	2.5a	39.0a	0.3a
	14	28	4.4a	2.5b	1.2c	5.5a	43.4a	0.2a
	28	42	3b	2.2b	10a	5.8a	53.0a	0.4a
	42		3.8	1.1	0.5	8.2	18.7	0.03
± SEM		3.9	1.1	0.5	8.2	18.7	0.03	0.1
L.S.D (5%)		5.5	2.3	0.7	24.4	39.6	0.6	0.8

Each treatment consisted of 10 cuttings and was replicated three times. Experiments were arranged in a complete randomized design. Cuttings were analyzed for starch, soluble and sugar alcohols by HPLC or spectrophotometer. Data were statistically analyzed using Genstat 5, 4.1 Release, 4th Edition, Lawes Agricultural Trust, IARC, Rothamsted. Treatment means followed by different letters (in a column) differ significantly from each other at the 5 % level of significance.

2.4 Discussion

2.4.1 Influence of IBA, rooting-cofactors, season on rooting of terminal and basal softwood, semi-hardwood and hardwood cuttings of three peach cultivars

The rooting percentage of peach cuttings of all three cultivars was significantly influenced by the IBA treatments in both terminal and basal positions. A trend towards an increase in rootability was observed with increasing levels of IBA application. IBA application enhances rooting by increasing free IBA, or by acting synergistical with IAA (Jarvis, 1986). IBA furthermore enhances tissue sensitivity to IAA and thereby increasing rooting ability (Haissig,1986). In cuttings taken on 25.10.1999 and 22.11.1999, rootability was positively influenced by the low concentration of IBA applied (500 mg l^{-1}) (Fig 2.1 B, 2.2 B and Fig 2.3). However, there was no significant difference between higher IBA concentrations and 500 mg. l^{-1} in percentage rooting (Fig 2.1 to Fig 2.3). This phenomenon can be explained by the tendency of softwood cuttings to root easily when propagated under intermittent mist after an auxin application. Furthermore, softwood cuttings have higher auxin and lower carbohydrate levels than dormant hardwood cuttings (Hartmann *et al.*, 1997). Hence, higher concentrations of exogenously applied auxin are needed in hardwood cuttings compared to softwood cuttings to initiate ARF in most species (Cuir *et al.*, 1993). An increase in auxin concentration from 500 mg l^{-1} to 1000 mg l^{-1} and 2000 mg l^{-1} improved rooting significantly (Fig 2.1 to Fig 2.3) particularly in in cuttings evaluated on the 04.05.2000 and 10.07.2000 (Fig 2.1 to Fig 2.3). According to Alvarez *et al.* (1989) the mode of action of IBA is the following: After application, IBA is transported from the cutting base to the upper part of the cuttings. Transport of IBA is faster or more efficient than transport of IAA. IBA is furthermore rapidly metabolized into IBA conjugates (Alvarez *et al.*, 1989). These conjugates are superior to free IBA in serving as an auxin source during root initiation (Wiesman *et al.*, 1989). In stem tissue, auxin is transported

strictly basipetally in the phloem (Jarvis 1986). Hence, application of synthetic auxins is made to the basal end of cuttings to simulate the natural downward-flow of IAA. However, it was earlier on found that basal applications of IBA result in better rooting than terminal applications (Bloch, 1943). When radioactively labeled IBA was used as an IBA source, most of the radioactivity remained in the basal part of the cutting, in both leafy and leafless cuttings (Bloch, 1943), implying that IBA was transported acropetally. Both types of cuttings (basal and terminal) absorbed similar amounts of IBA, indicating that transpirational 'pull' is not the main cause of IBA absorption and translocation.

Four phenolic rooting-cofactors were evaluated with respect to their ability to influence rooting of peach cuttings in the presence of differing IBA concentrations (Fig 2.4 to Fig 2.15). Phloroglucinol (Fig 2.24, 2.28 and 2.12) and chlorogenic acid (Fig 2.5, 2.9 and 2.13) improved rooting significantly (P=0.05) throughout the study period. These results confirm findings by Heuser and Hess (1972), who showed that chlorogenic acid and phloroglucinol act synergistically with IAA to promote rooting. Quercitin (Fig 2.6, 2.10 and 2.14) and rutinin (Fig 2.7, 2.11 and 2.15) did not influence the rooting of peach cuttings in the presence of IBA significantly (P=0.05). The non-effectiveness of these compounds might have been due to the sufficient mechanism possibly triggering ARF (Wiesman *et al.*, 1989). These results confirm the suggestion by Davis and Haissig (1990), that small concentrations of phenoxy-compounds such as phloroglucinol and chlorogenic acid added to either IBA or NAA increase ARF to a larger extent than phenoxy-compounds alone. Phenolic compounds play a role in ARF protecting the root-inducing IAA from destruction by the enzyme IAA oxidase. It has been suggested by Wilson and van Staden (1990), that the action of rooting promoters and inhibitors is controlled by chemical processes within the plant. Irrespective of their

chemical identity, "lower" concentrations promote rooting and "higher' concentrations are inhibitory. According to these authors phenolic compounds applied exogenously, can interact with the endogenous IAA metabolism. Figures 2.4 to 2.15 show that significant interactions exist between presence of phenolic compounds and rooting of peach cuttings. The σ-dihydroxy compounds such as chlorogenic acid and pholoroglucinol, have been found to enhance ARF in most *Rosaceae* species by inhibiting IAA oxidase (Morsink and Smith, 1975).

Differences were observed in the ability to form adventitious roots in terminal versus basal cuttings of all three peach cultivars studied. In summer and early autumn, in all three cultivars, cuttings prepared from terminal cuttings obtained significantly (P=0.05) higher rooting percentages than their basal counterparts (Fig. 2.1 to 2.3). These findings confirm results obtained by Marini (1983), who observed that terminal softwood and semi-hardwood cuttings of 'Red Heaven' and 'Reliance' peach rooted better than their basal counterparts. The high rooting ability of terminal softwood and semi-hardwood cuttings has been attributed to higher auxin levels in terminal opposed to basal parts of the cutting. On the other hand, higher rooting ability of basal than terminal hardwood cuttings has been attributed to the accumulation of carbohydrates in the cutting bases combined with an accumulation of IAA in the basal part due to its basipetal polar transport (Bal and Sandhawalia. 2000).

A seasonal variation in rooting of peach cuttings was observed in all three cultivars. Except for 04.05.2000 and 10.07.2000 cuttings, all IBA concentrations on both terminal and basal cutting positions, affected rooting significantly (P=0.05). Highest rooting percentages were obtained during

the shoot flush in spring (October and November), and lowest rooting ability was observed in leafy autumn (May) and leafless winter (July) cuttings. This seasonal variation in the ease of rooting of cuttings has been correlated to the shoot RNA level, which gives an indication of bud activity (Hartmann *et al.*, 1997). Davies (1984) fond high levels of shoot RNA and increased vascular cambial activity during peak rooting periods in various woody plant species. In the presented study rooting-cofactors and IBA concentrations increased rooting throughout the different seasons (Figures 2.4 to 2.15). However, phloroglucinol and chlorogenic acid attained higher rooting percentages (P=0.05) than rutinin and quercitin. Therefore, a possible increase in shoot RNA which stimulating protein and nucleic acid synthesis, followed by an increase in a vascular cambial activity, may have occurred during October and November, the active growth period of peach trees. Seasonal effects on rootability of the cuttings can not be viewed without considering the response of the cuttings to the environmental conditions at different times of the year or without the physiological processes correlated to the mother plants. Low rooting ability in summer (Fig 2.4 to 2.15 D) and late winter (Fig 2.4 to 2.15 F) is associated with newly expanding buds and shoots, which act as sinks for carbohydrates and phytohormones (Issel and Chalmers, 1979). Seasonal changes in rooting response of stem cuttings of temperate-zone species such as *Prunus domestica* and *Pyrus communis* have been found to be triggered by endogenous growth substances (Brar *et al.*, 1982) and are correlated to the production and accumulation of photosynthates (Bal and Sandhawalia, 2000).

2.4.2 Influence of IBA and Ca-EDTA on rooting of "wounded" and "non-wounded" peach hardwood cuttings

Treatment with IBA was virtually essential to elicit a wounding response (Table 2.1). This response could be explained by an endogenous auxin level too low to induce a rooting response (Hartmann *et al.*, 1997). Howard *et al.* (1984) showed that wounding the cutting bases *of Malus domestica*, consistently, and often greatly increased the number of rooted cuttings and the number of roots produced per cutting compared to a simple removal. Figures 2.1 to 2.3 show that the number of roots per cutting tended to increase with increasing IBA concentration (from 0 mg l^{-1} IBA to 2000 mg l^{-1} IBA) applied to the cuttings. Furthermore a tendency towards a higher number of roots per cutting was evident in "wounded" than in "non-wounded" cuttings (Table 2.2). There was also considerably more callus formation along the longitudinal axis of the wound/incision than at the cut base of the cutting during rooting. It is therefore possible that when cutting the stem transversely, more cambial cells are cut across their longitudinal axis, and these damaged cells are induced to form callus .

It appears that wounds create areas capable of initiating roots, which then develop only due to the stimulatory effects of a root-promoting hormone (e.g. IBA). Howard (1973) found improved rooting if an IBA solution was applied to a "wounded" cutting base. He also suggested that the effect resulted from enhanced uptake of the IBA solution through the incision by capillary action (Howard. 1971).

The role of Ca-EDTA ni ARF is not fully understood. According to Taiz (1991) Ca-EDTA can open transpiration channels, thereby increasing auxin basipetal transport. However, Ca-EDTA increased the rooting percentage only when applied to the "wounded" cutting together with IBA (Table 2.1). Hence, Ca-EDTA might have increased rooting through improved IAA-Ca antiport, resulting in a basipetal transport of IAA rather than through action via IBA (Marschner, 1998). Furthermore, a significant influence of Ca-EDTA on rooting when applied to "wounded" cuttings was observed, suggesting the ability of Ca-EDTA to react with endogenous IAA in the process of ARF.

2.4.3 Role of sugars in softwood peach cuttings during the adventitious rooting process

Changes in carbohydrate levels during the ARF process (Table 2.2) underline the importance of sugars, sugar alcohols and non-structural carbohydrates during this process. The high content of sugars at the first sampling date in spring (04.10.00) may have been caused by the high metabolic activity of softwood cuttings, making the bound and stored carbohydrates readily available (Haissig, 1984). Cuttings not treated with IBA showed a trend towards carbohydrate depletion, especially in sucrose and starch from 0 to 42 days in the mistbed. This might have been due to the use of these sugars in the respiration process. Softwood cuttings treated with IBA (Fig 2.1 to Fig 2.3) were characterized by a higher rooting percentage than untreated ones. Hence, once the root and shoot system developed, cuttings seem to synthesize photosynthates and as a result, an accumulation of sugars was observed in cuttings after 28 and 42 days in the mistbed. Furthermore, tissues from cuttings treated with 1000 mg l^{-1} IBA displayed no sugar consumption by the developing roots during ARF (Table 2.2). It is, however, believed that during cutting preparation there was low metabolic

activity within the cuttings due to stress, and with the subsequent placement of the cuttings in the mistbed the metabolic activity resumes and this leads to sugar consumption. It is therefore, believed that in tissues treated with IBA less or no plant sugar reserves were used in the ARF process (Hatzilazarou, 2000). During later stages of the ARF process, developing root primordia and root initials act as sinks, and a net sugar consumption is observed (Harman *et al.*, 1997). The optimal carbohydrate concentration in stock plants and rooting of cuttings has not been defined (Gill, 1995). Attempts to associate total carbohydrate concentration or specific carbohydrates to the metabolic control of rooting have also not been successful (Hatzilazarou, 2000). This is attributed to the difficulty to quantify the relative proportions of total or specific carbohydrates necessary to induce rooting (Hatzilazarou, 2000).

Cuttings accumulate soluble carbohydrates and starch at their bases prior to formation of root primordia (Gill, 1995). When root initiation is not limited by factors other than local availability of sugars, enhanced transport of sugars from the leaves to the rooting region has been demonstrated during early stages of root initiation (Gill, 1995). ARF is believed to be dependant on sugar transport via the phloem, irrespective of the sugar content of the surrounding tissues (Haissig, 1983). It is therefore believed that auxin application readily enhances transport of sucrose from leaves to the bases of the cuttings where the process of root primordia and root initiation occurs (Hartmann, *et al.*, 1997). Inclusion of Ca-EDTA in rooting solutions of IBA has however, resulted in improved results in peach cuttings, though its mode of action in stimulating rooting alone or in combination with IBA is not well understood.

CHAPTER THREE

Anatomy of ARF in hardwood cuttings of *Prunus persica* 'Earlibelle'

3.1 Introduction

Two patterns of ARF have been recognized in cuttings of herbaceous and woody species (Qrunfleh *et al.*, 1992). ARF can either originate from root primordia in cells associated with or in close proximity to the vascular system, or from indirect formation of adventitious roots with an interim period of undifferentiated cell divisions, initiated in the parenchyma or epidermal cells. In some species or under certain environmental conditions, the patterns may be consecutive. Selected cells from these subsequent cell divisions eventually organize to initiate adventitious root primordia (Harbage *et al.*, 1994). In general, herbaceous species and 'easy-to-root' woody species form adventitious roots through the direct pattern of root formation, while species that are 'difficult-to-root' form roots through the indirect pattern (Preece, 1993).

The ARF process is divided into two stages, i.e. root initiation and root growth. These two stages vary among species and cultivars. Plant age and organ type or plant part also respond variously to adventitious rooting (Lovell and White, 1986). Root initiation normally starts with an induction and activation phase which involves cell division, followed by root primordia development from *in situ* divisions of phloem parenchyma and cambial cells (White and Lovell, 1984). Those cells that are activated initially may or may not be the ones that become primordia. Early root initiation is characterized by the appearance of cells with a large centrally located vacuole. These cells can potentially give rise to the initials of the organized root tip (Geneve, 1991).

According to Haissig and Davis (1994) the fundamental basis of adventitious root formation in

"woody" and "herbaceous" plants is the least understood of all plant developmental processes. The primary chemical stimulus for differentiation and root initial formation (the critical steps of adventitious root formation), as well as subsequent organization of meristemoids (meristematic centers of cells actively dividing into root primordia) remains unknown (Hartmann *et al.*, 1997). Studies by Arafeh (1990) and Arafeh *et al.* (1991) indicate that ARF in the peach cultivars 'Floridabelle' and 'Floridagold' occurs from wound roots (roots developing from wounded cutting bases) and can take place in internodal as well as nodal areas, arising either externally from callus tissue or internally from cambium, phloem and/or cortex tissue.

Although auxins are commercially applied to increase rooting percentage their effect on root initiation has not been studied in detail (Geneve, 1991). Therefore, the anatomical changes in peach cuttings during rooting as influenced by the auxin treatment, were investigated in this study. Furthermore, possible sites of root origins in peach were examined by transverse sections of stem cuttings in various stages of root formation using Transmission Electron Microscopy (TEM) and light microscopy (LM). TEM was used to characterize anatomical changes in the vicinity of vascular bundle and surrounding cells during rooting, whilst LM was employed to observe changes in tissue structure that might have an influence on rooting of peach cuttings.

3.2 Materials and Methods

3.2.1 Plant material

To evaluate the influence of IBA on the anatomy of ARF, hardwood cuttings of 'Earlibelle' peaches were collected on 5/06/2000. 'Earlibelle' peach cuttings were used in this study because in previous studies on rooting of cuttings (chapter 2) 'Earlibelle' displayed the lowest rooting percentage of hardwood cuttings of the three cultivars tested. Detailed observations of cell

differentiation, cell wall structure and the cells around root initiating regions were made. Hardwood cuttings of 'Earlibelle' were examined after zero, four, and six weeks of root induction treatments with 0, 4000 and 6000 mg l^{-1} IBA.

3.2.2 Transmission electron microscopy

Plant tissue preparation was carried out according to Anderson and Andre (1968). The fresh material was fixed for 8 hours in 3 % glutaraldehyde (buffered to 7.2 pH with 0.05 M sodium cacodylate buffer) (Agar Scientific Ltd, Essex, England), followed by two washes of 30 minutes each in the same buffer. Thereafter tissues were fixed for two hours in 2 % osmium tetroxide (Electron Microscopy Sciences, Washington, USA), dehydrated in an ethanol series (Appendix 7) and finally embedded in epoxy resin (LAA Research Industries INC., Burlington, USA). Sections of 60ηm were cut on a Ultramicrotome LKB111 (Ted Pella INC., California, USA), then stained with 2 % uranyl acetate and lead citrate, followed by examination in a Philips CM 120 BioTwin TEM (Industrial and Electro-acoustic Systems, Eindhoven, Holland) operating at an accelerating voltage of 100kV.

3.2.3 Light microscopy

Hardwood cuttings of 'Earlibelle' were planted in the mistbed for rooting as previously described. Samples were taken at zero, four, and six weeks after root induction treatments. Basal portions of cuttings were cut into 1 cm pieces. The samples were fixed according to the method of Johansen (1940) in FAA (formalin: acetic acid: ethanol: water, 2:1:10:7, v/v/v/v), then dehydrated in a graded ethanol/tertiary-butanol series (Appendix 7 a) and finally embedded in wax (Appendix 7 b) (Johansen, 1935) (all chemicals supplied by Merck Laboratory Suppliers Pty Ltd, Johannesburg, South Africa). Embedded tissue was mounted on wooden blocks and cut into 15

to 18 μm sections on a 45 Reichert rotary microtome (Lipshaw Manufacturing Company, Detroit, Michigan, USA). Sections were mounted on slides with Haupt's adhesive (Haupt, 1923), stained with Safranin and Fast Green (Sass, 1958) (Appendix 7 c) and covered with cover slips before examination using an Olympus BH-2 light microscope (Olympus Opticle Company Ltd, Tokyo, Japan)

3.3 Results

3.3.1. Light microscopy studies

Untreated cuttings showed little or no meristematic cell development during rooting (Plates 1 A and 1 B). At the time of cutting separation from the mother plant, no distinct cell division was visible in the basal cutting tissues (Plate 1 A). After six weeks in the mistbed transverse sections of the cuttings revealed little changes in shape and enlargement of cells and only minor appearances of interfascicular regions were observed, extending towards the interior of the pith (Plate 1 B). Cuttings treated with 4000 mg l^{-1} IBA on the other hand, showed significant changes in their structure after two, four and six weeks in the mistbed (Plates 2 to 5). There was little or no activity in cuttings treated with 4000 mg l^{-1} after separation of the cuttings from the mother plant (Plate 2). Two weeks after cuttings were prepared and dipped in 4000 mg l^{-1} IBA the differentiation of secondary xylem cells was observed (Plate 3). Furthermore, from two to four weeks in the mistbed an increase in cell division was noted, which resulted in destruction of positional cambium due to cortical cell enlargement (Plate 3). There was little evidence of cell division and meristematic activity after four weeks in the mistbed (Plate 4). Six weeks after cutting treatment, root primordia were visible. However, the site of root initiation was not yet clearly identifiable (Plate 5). Periclinal divisions accompanied by radial expansion resulted in root primordia formation (Plate 5). Most protophloem sieve elements are believed to have been

crushed by the enlarging cortical cells responding to ABA treatment and partly by intruding primary root primordia from dividing cells.

Treatment of peach cuttings with 6000 mg l^{-1} IBA also resulted in the development of root primordia and, therefore, adventitious rooting (Plate 6). Cuttings treated with this IBA concentration showed little cell division and enlargement after two weeks (Plate 7). After four weeks in the mistbed, the cuttings showed an increase in size of the meristematic tissue into cortical cells compressing these in the process (Plate 8). Pith cells did not undergo any cell division or apparent enlargement, hence, pith did not increase in size (Plate 8). Six weeks after treatment planes of cell division were oriented in many directions and the meristematic cells were densely vacuolated (Plate 9). Meristematic regions separated by files of radially elongated cells were identified as root primordia (Plate 9).

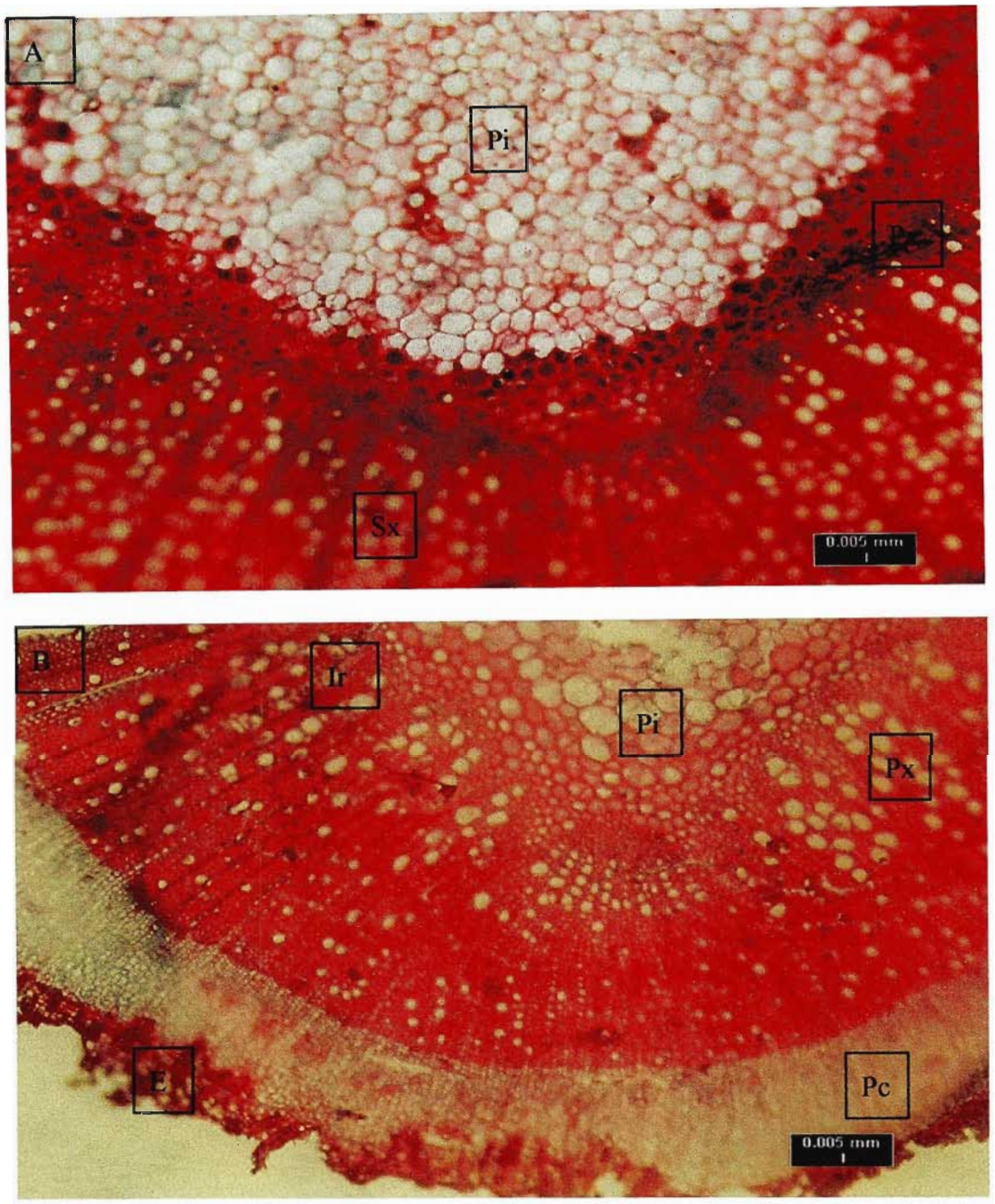


Plate 1 A and 1 B: Transverse sections of untreated 'Earlibelle' peach cuttings after separation from the mother plant (1 A) and after 6 weeks in the mistbed (1B); magnification (1 A) = x40 and (1 B) = x100. Abbreviations: epidermis (E), interfascicular region (Ir), pith (Pi), primary xylem (Px), secondary xylem (Sx) and positional cambium (Pc)

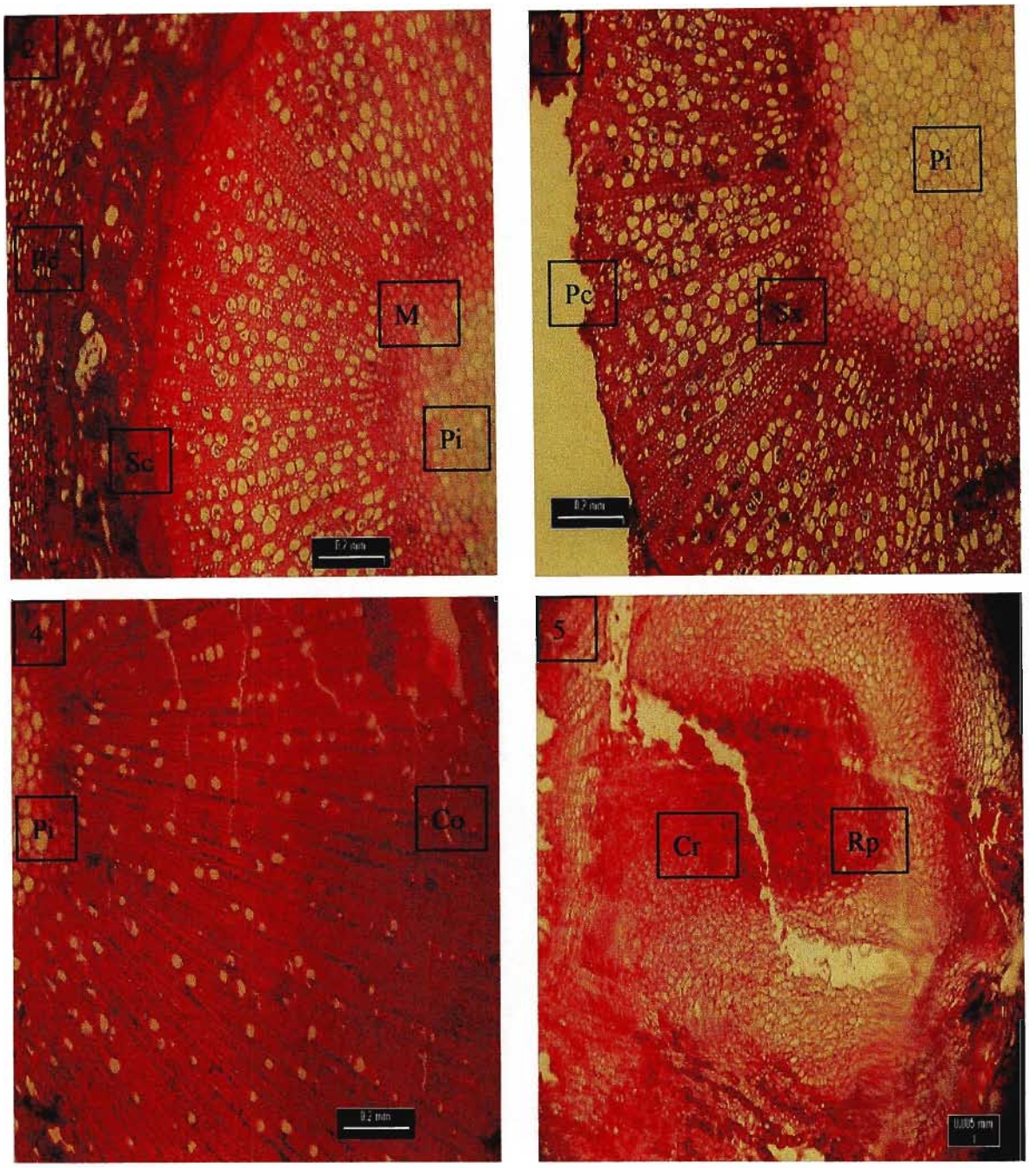


Plate 2 to 5: Transverse sections of 'Earlibelle' peach cuttings treated with IBA (4000 mg l^{-1}) after separation of cuttings from mother plant (Plate 2), after 2 weeks (Plate 3), after 4 weeks (Plate 4) and after 6 weeks in the mistbed (Plate 5). Magnification; (2) = x100, (3) = x100, (4) = x40 and (5) = x1000. Abbreviations: pith (Pi), root primordium (Rp), metaphloem (M), sclerenchyma caps (Sc), positional cambium (Pc), cortex (Co),cambial region (Cr) and secondary xylem (Sx)

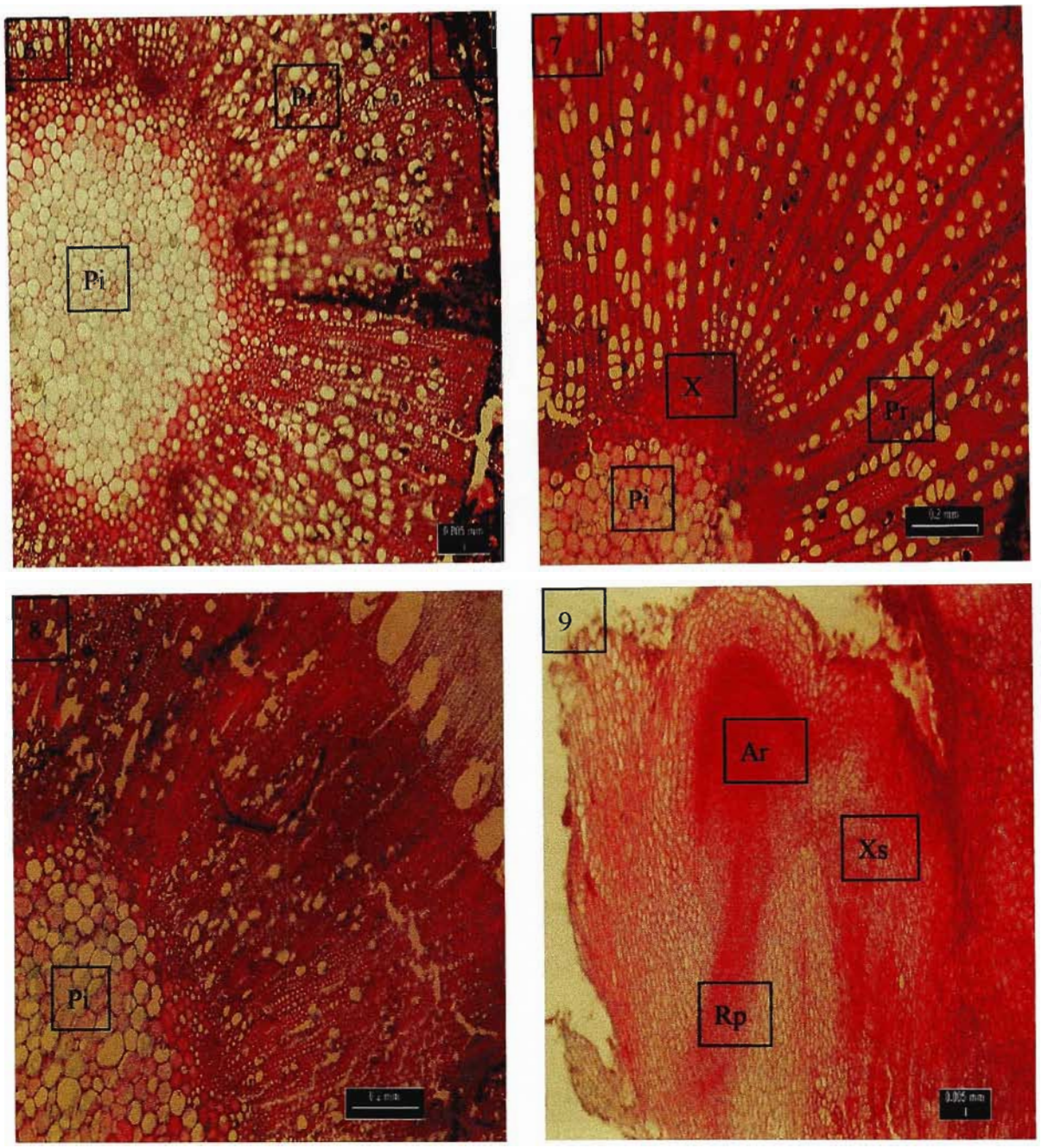


Plate 6 to 9: Transverse sections of 'Earlibelle' peach stem cuttings treated with 6000 mg l^{-1} IBA after separation from the mother plant (Plate 6), after 2 weeks (Plate 7), after 4 weeks (Plate 8) and after 6 weeks (Plate 9) in the mistbed. Magnification; (6) = x100, (7 & 8) = x100 and (9) = x1000. Abbreviations: pith (Pi), xylem strand (Xs), root primordium (Rp), adventitious root (Ar), epidermis (E), phloem rays (Pr), xylem (X)

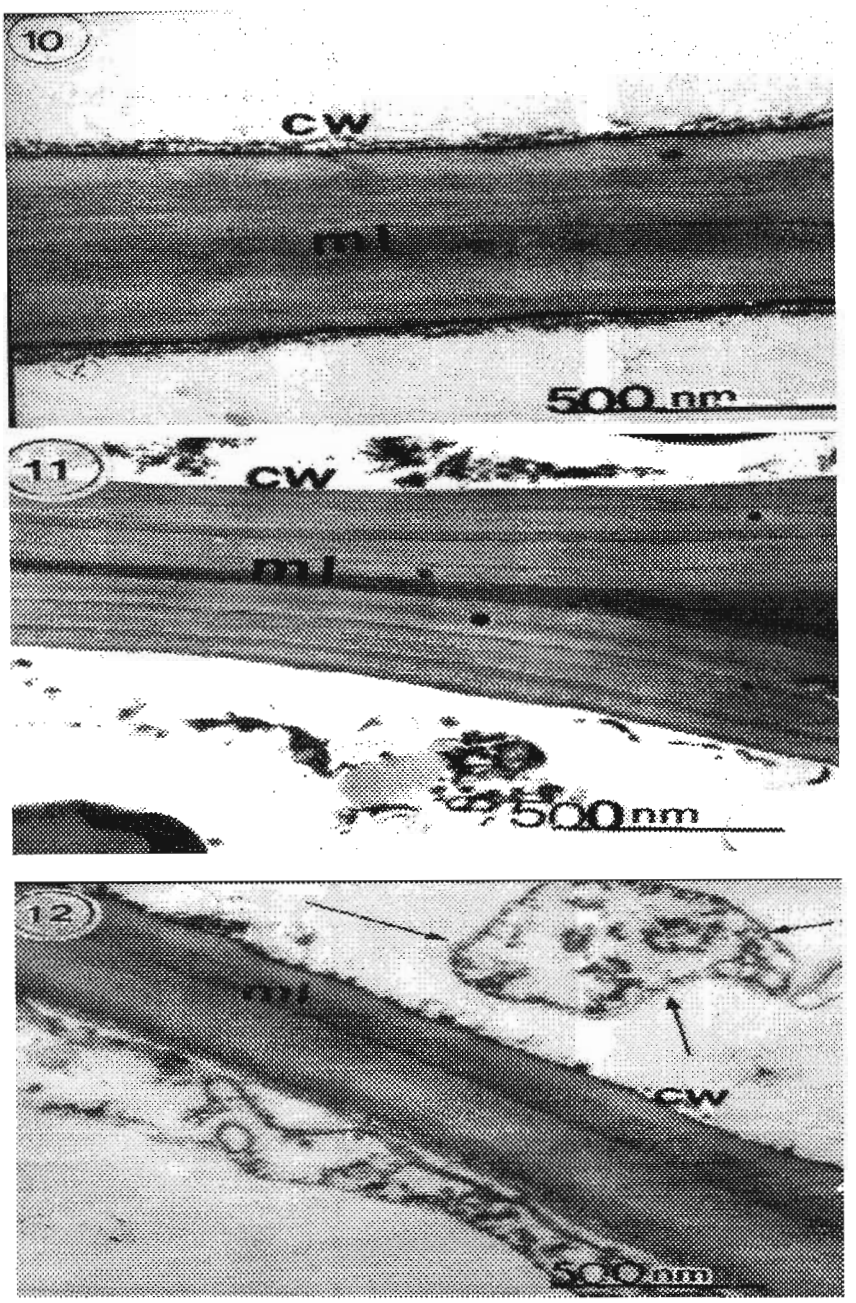
3.3.2 Transmission electron microscopy

Light microscopy studies of peach cuttings treated with IBA showed that cortical cells and cells surrounding these are involved in ARF. TEM studies revealed that cortical cell walls from untreated cuttings are characterized by thick walls (Plate 10) and that cells from treated cuttings show shrinkage as well as rupturing of their structure (Plate 11). After six weeks in the mistbed, cuttings treated with 4000 mg l^{-1} IBA showed cell wall rupturing (Plate 12), possibly due to tracheary elements developing into outgrowths, which may later form root primordia. Tracheary elements were already visible after two weeks and clearly after six weeks in the mistbed in tissue treated with 4000 mg l^{-1} IBA (Plate 13 and 14). 'Earlibelle' cuttings treated with 6000 mg l^{-1} IBA, showed short and oblique shaped cortical cells (Plate 15). Cuttings left for six weeks in the mistbed after 6000 mg l^{-1} IBA treatment, showed cell elongation and thickening of cortical cell walls (Plate 16). Development of fibre sclereids was evident after two and six weeks following the treatment of the cuttings with 4000 mg l^{-1} IBA (Plate 17) and 6000 mg l^{-1} IBA (Plate 18). The fibre sclereids extended in all directions within the cortical cells, from the interior of the cortical cells towards the cambium cells. Cuttings treated with 4000 mg l^{-1} IBA did not show any degeneration in plastid cells after two weeks in the mistbed (Plate 19), whilst the plastid cells within the cortical cells (Plate 20) showed remarkable degeneration during the rooting process after six weeks in the mistbed. Tissues of cuttings treated with (4000 and 6000 mg l^{-1} IBA) displayed occurrence of meristematic tissue, cortical cell wall rupturing and plastid cell degeneration after an extended period in the mistbed.

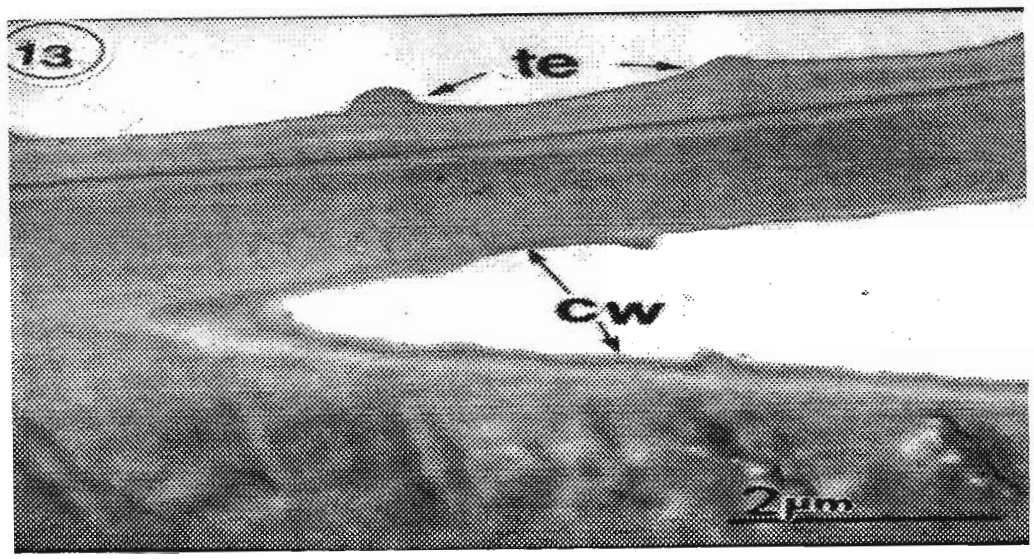
0 mg l^{-1}

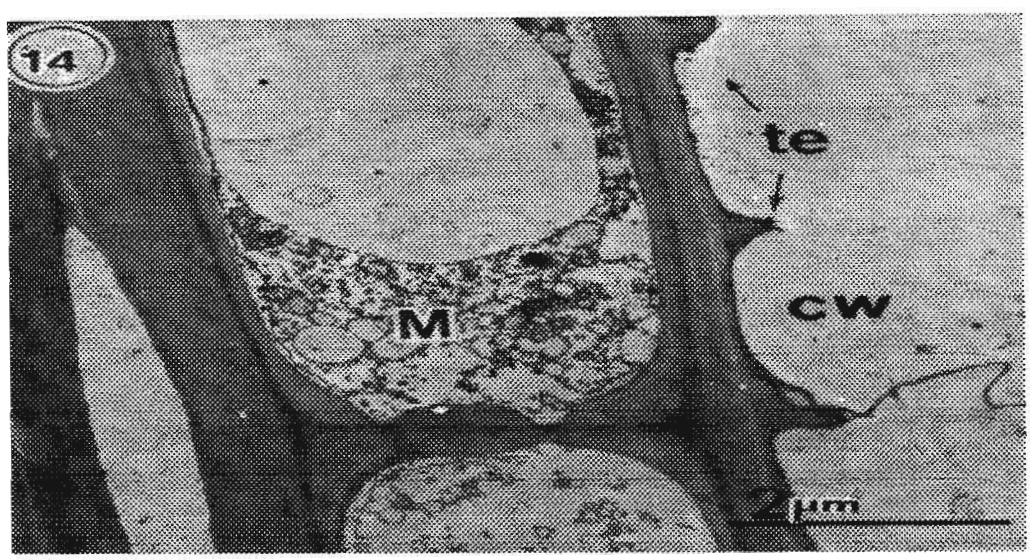
4000 mg l^{-1}

4000 mg l^{-1}

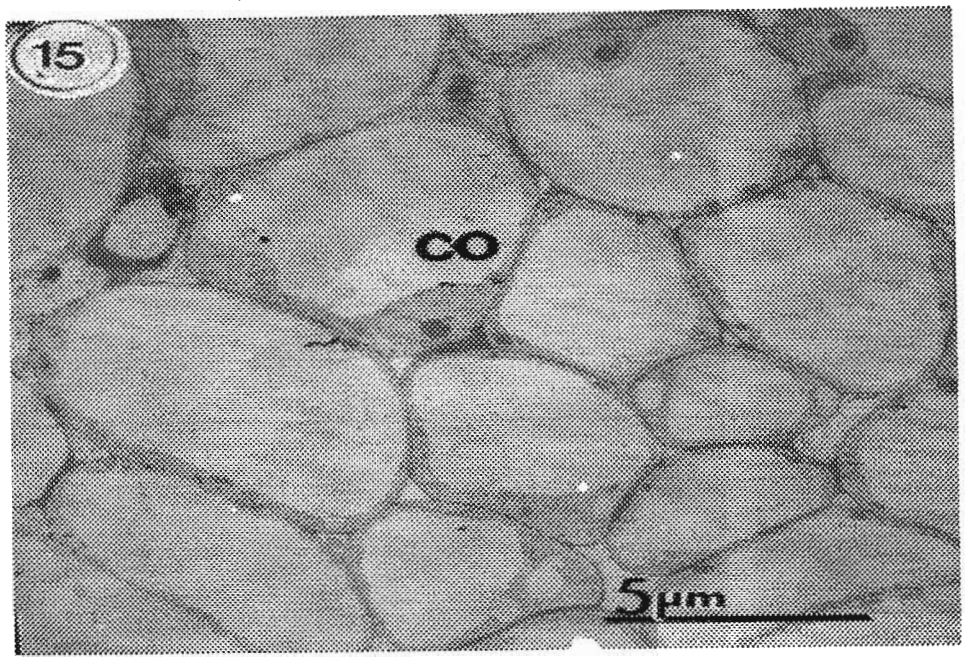


Plates 10, 11 and 12: Cell walls of 'Earlibelle' peach cuttings at the time of IBA treatment (10), four weeks after treatment with IBA (11) and six weeks after placement in the mistbed (12). Abbreviations: cell wall (cw) and middle lamella (ml). Arrows indicate regions of cell wall rupturing

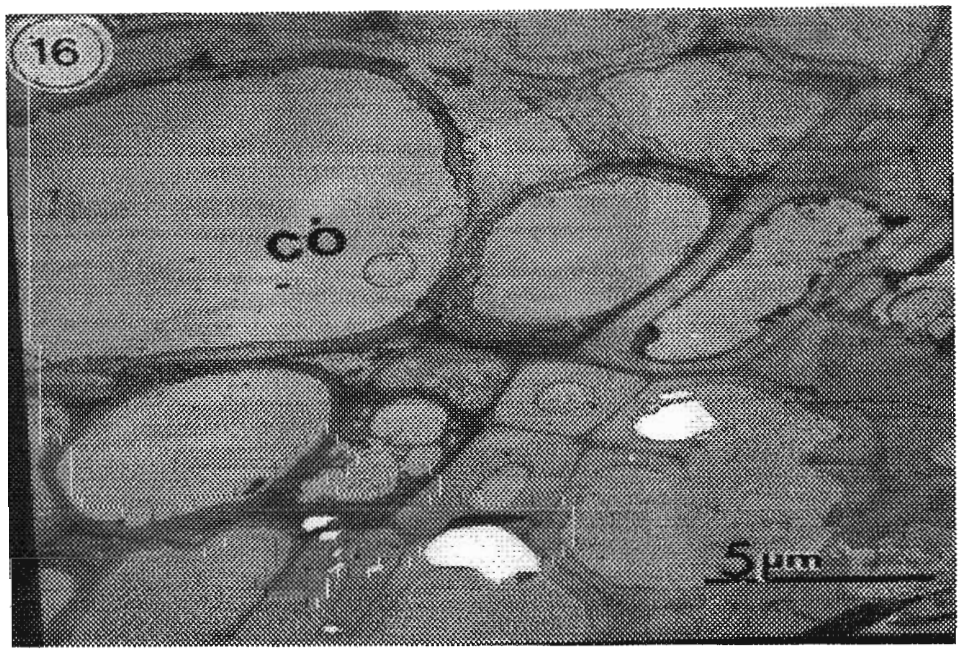




Plates 13 and 14: Emergence of tracheary elements of 'Earlibelle' peach cuttings after 4000 mg l^{-1} IBA treatment and subsequent placement for two (13) or six weeks (14) in the mistbed. Abbreviations: cell wall (cw), metaphloem (M) and trachery elements (te)

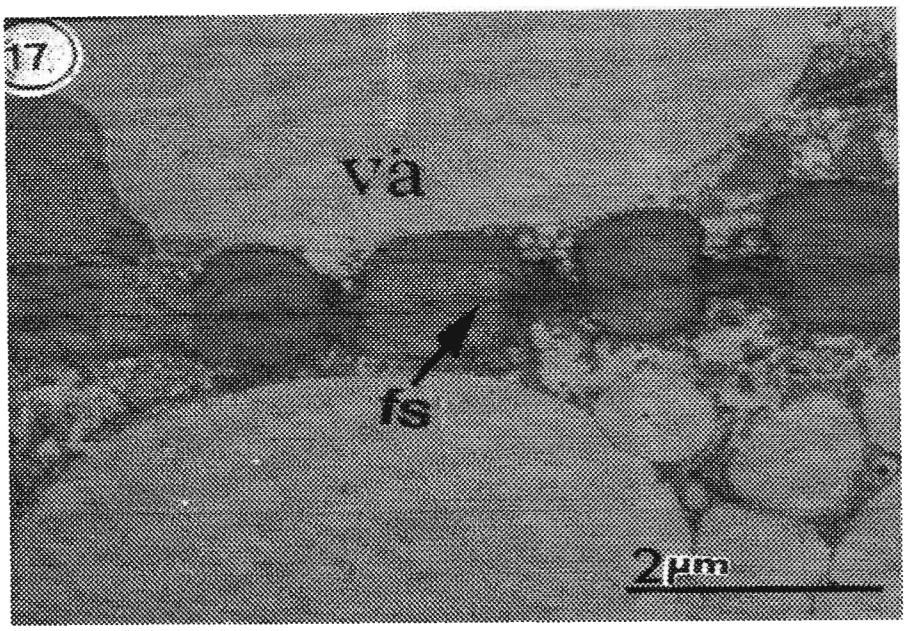


0 weeks

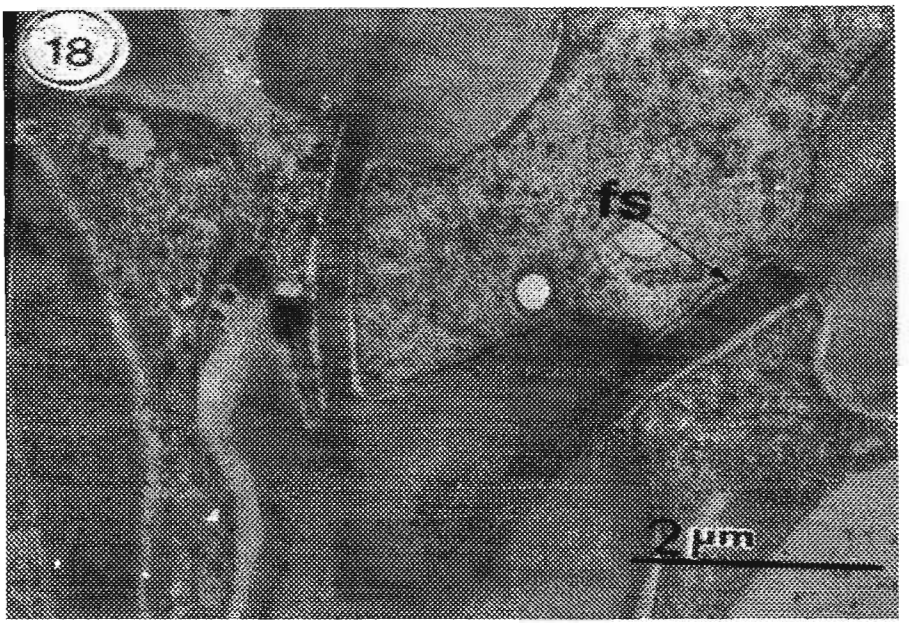


2 weeks

Plates 15 and 16: Cortical cells of 'Earlibelle' peach cuttings at the time of IBA treatment (6000 mg l^{-1} IBA) and after two weeks in the mistbed. Abbreviation: cortex (co)

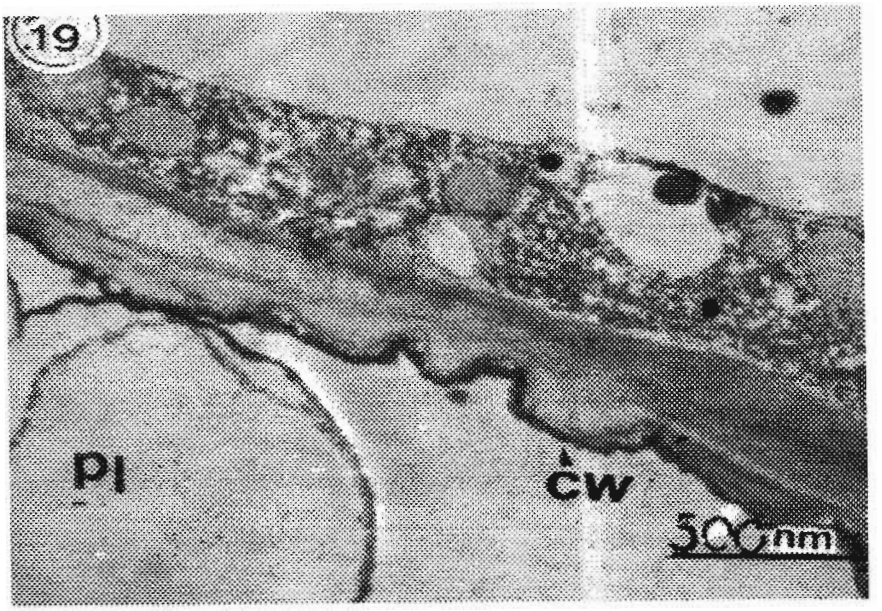


2 weeks (4000 mg l^{-1})

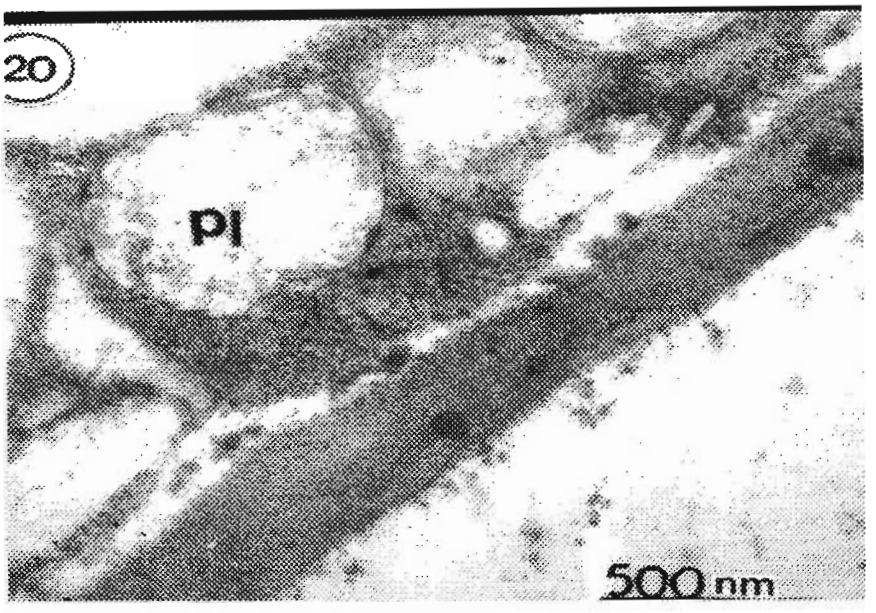


6 weeks (6000 mg l^{-1}IBA)

Plates 17 and 18: Development of fibre-sclereids in 'Earlibelle' stem cuttings after treatment with 4000 and 6000 mg l^{-1} IBA and subsequent placement in the mistbed. Abbreviations: fiber-sclereids (fs) and vacuole (va)



2 weeks (4000 mg l^{-1} IBA)



6 weeks (6000 mg l^{-1} IBA)

Plates 19 and 20: Degeneration of plastid cells within cortical cells in 'Earlibelle' stem cuttings after 4000 and 6000 mg l^{-1} IBA treatments and subsequent placement in the mistbed. Abbreviations: plastid cell (pl) and cell wall (cw)

3.4 Discussion

Light microscopy studies showed that cells of the cortex and cells within the vicinity of the cambial region are involved in adventitious root formation in peaches (Plate 9). This was indicated by the degree of meristematic development and cell division in these cells (Plate 9),a feature typical for root primordia occurrence according to Haissig and Davis (1994). It is believed, however, that root primordia initiation in cuttings requires a polar transport of an auxin-like substance from the shoot tip to the base of the cutting (Jarvis, 1986), with the auxin-like substance moving down leaf traces and accumulating at the base of the severed shoot, where it triggers root formation (Hartmann *et al.*, 1997). 'Earlibelle' peach cuttings that were not treated with IBA did not show any changes in their cell structure and no development of meristems was observed. Application of 4000 and 6000 mg l^{-1} IBA resulted in cell size changes and enlargement in tissues which did not display such changes before (Plate 8 and 9). Cells of the cambial region became actively involved and developed into secondary xylem and phloem rays (Plates 2 to 5). Most of the xylem and phloem rays were possibly crushed by the enlarging cortical cells and partly by intruding primary root primordia (Plate 5) from dividing cells, such events have been described by Harbage *et al.* (1993). TEM studies revealed that initiation of cell division by the rooting treatment took place in the cambial region and in cortical cells and yet only divisions in the cortical cells seemed to result in meristematic regions leading to development of tracheary elements, which activate root primordia in woody species (Esau, 1961) (Plate 13 and 14). As cortical cells enlarged, there was evident cell wall shrinkage and rupturing. The phenomenon of root primordia extending and forcing its way through cortical cells has been described by Doud and Carlson (1997). Higher concentrations of IBA (4000 and 6000 mg l^{-1}) resulted in root initial development, rupturing of cortical cell walls, development of fiber-sclereid and plastid cell degeneration, whilst untreated cuttings did not show such changes. Although other

undifferentiated cells (i.e. parenchyma cells of pith, xylem, cambial region and phloem) reacted to the treatment by cell division, root primordia could only be identified in cortical cells region.

CHAPTER FOUR

Influence of culture media, temperature, photoperiod and stratification period on *in vitro* growth and development of peach embryos

4.1 Introduction

Tissue culture is a collective term to describe a variety of *in vitro* plant cultures. Strictly, it refers to cultures of organised cell aggregates. In practice the following types of cultures are recognised: callus (tissue) culture, suspension (or cell) culture, protoplast culture and anther culture (George, 1993). In addition, embryo culture can assist in the rapid production of seedlings from seeds that have a protracted dormancy period and it enables seedlings to be produced when a certain genotype resulting from some interspecific crosses conveys a low embryo or seed viability. Early-ripening genotypes of *Prunus persica* often have small embryos after fruit maturity. These embryos are difficult to germinate *in vitro* and show partial or disordered growth (Scozzoli and Pasini, 1991). Therefore, embryo culture has been used by breeders to achieve germination of embryos from such early-ripening peach cultivars. This overcomes a great handicap to breeding, since one of the most important objectives of peach breeding programmes is to obtain very early-ripening cultivars as it allows regeneration of plants which can not be easily grown from seeds. Embryos of such cultivars often are underdeveloped and dormant at fruit maturity and require a certain after-ripening period in a culture medium before they can overcome dormancy and germinate. Embryo culture is in this case necessary to achieve germination (Antonelli *et al.*, 1988).

The successful development of an embryo into a plant depends on many factors. Embryos of some species are easier to grow in culture than others. The plant genotype greatly influences the

culturing success (Rizzo *et al.*, 1998), but differences can occur even between closely related cultivars.

Furthermore, the ability of a peach embryo to germinate is correlated to its size (Ramming, 1990). Although embryo length and fresh weight are usually used as size measurements, embryo dry weight is a more reliable indicator of its maturity and viability (Foud *et al.*, 1995). Successful embryoculture depends, however, to a large extend on the medium composition during the establishment stage Rizzo *et al.* (1998). Such a culture medium usually consists of a solution of salts supplying the major and minor elements necessary for plant growth, together with various vitamins, amino acids and a carbon source (usually sucrose) (Hammerschlag *et al.*, 1987). Sucrose has been found to be a better carbon source than either glucose or fructose for tissue culture (Scozzoli and Pasini, 1991). Sucrose is involved in the two types of morphogenisis defined by Hicks (1980) as, "the development of organs from differentiated cells of a newly-transferred piece of whole-plant tissue, without proliferation of undifferentiated tissue" , as well as in indirect morphogenesis, according to George (1993), "the development of organs from unspecialised, unorganised and dedifferentiated tissue". Although sucrose acts mainly as a carbon source, part of its effect seems to be also due to osmoregulatory activity providing a suitable water potential for embryo germination (Mukherjee *et al.*, 1991). Furthermore, addition of amino acids or plant growth regulators to the culture medium may stimulate embryo growth. GA_3 has been found to improve growth of the meristematic tissue of certain dicotyledonous species and can help to prevent callus formation when explants are cultured on a medium containing auxins and cytokinins (Vine, 1968). When added to the medium as the only growth regulator, GA_3 can stimulate leaf and shoot development from apical domes of *Chrysanthemum* (Vine, 1968). Addition of either casein hydrolysate, amino acids or yeast extract to a dilute medium (quarter or half strength) with low levels of inorganic nitrogen can also improve embryo growth. Casein

hydrolysate is a complex mixture of 18 amino acids that has been widely used as an adjunct for embryo culture media (Rizzo *et al.*, 1998). Several media formulations have been used for embryo culture, such as: Murashige and Skoog Medium (Murashige and Skoog, 1962), Modified Knop's Medium (Chapparro and Sherman, 1994), Woody Plant Medium (Lloyd and McCowan, 1981), Stewart and Hsu Medium (1978) and Shives Medium (Schenk and Hildebrandt, 1972).

Plant organs and tissues have to be located on the surface of a culture medium to be able to develop a root and shoot system. This is achieved by adjusting the medium viscosity with the help of gelling agents (Pan and van Staden, 1999). Ideally, the medium should be able to be sterilized by autoclaving, be liquid when hot and viscous when cool (George,1993). Agar has traditionally been used as a gelling agent for tissue culture and is still widely employed for the preparation of culture media. The contact between tissue and medium can be altered by varying the agar concentration from 0.7-1.0 % (w:v) (Rahman *et al.*, 1992). Lower or higher concentrations of agar either do not support explants or may lead to 'glassy tissue' development (De Lange, 1989).

Plant growth substances, especially auxins, are generally added to the medium to induce callusing in the explants (Irvine *et al.*, 1983). In cell and organ cultures auxins promote cell dispersion while cytokinins tend to cause cell aggregation. The relatively high level of auxins added to a medium for this purpose will prevent development of organs and organised tissues but if applied in embryoculture induces embryo development and formation of both, a shoot and a root system, in the culture (Hillman and Gaston, 1961). A high cytokinin to auxin ratio is generally required for direct induction of shoots and explants (George, 1993). By contrast, rhizogenesis usually follows a supply to the growing medium with auxin alone, or culturing in

a medium containing a higher concentration of auxins than cytokinins (Friedman *et al.*, 1985).

Besides adding plant growth substances to the medium, variation of the day length to the plant tissue culture can also influence the concentration of natural growth substances in the cultured tissue (Cathey and Campbell, 1980). Plants grown under long day usually exhibit a higher endogenous auxin concentration than those grown under short day conditions, regardless of the photoperiod requirement of the plant for flowering (Hillmann and Galston, 1961).

Lastly, media pH also affects the success of culturing explants. Many plant cells and tissues tolerate a pH of the culturing medium of about 4.0-7.2 (Butenko *et al.*, 1984). Best results of tissue and organ growth have, however, been obtained under slightly acid conditions. The uptake of charged anions is favoured at acid pH, while that of cations is best when the pH is close to 7 (Friedman *et al.*, 1985). The relative uptake of cations and anions by the tissue cultured *in vitro* alters the pH of the medium. The release of hydroxyl ions from the plant in exchange for nitrate ions take up from the medium results in a more alkaline medium (Fuggi *et al.*, 1981). The pH shifts caused by uptake of nitrate or ammonium ions during culture can lead to nitrogen deficiency if either nitrate or ammonium is used as the sole source of nitrogen without the addition of a buffer (Hyndman *et al.*, 1982).

The dormancy period of seeds and buds of temperate fruit trees is strongly and positively influenced by the chilling temperature, with effective temperatures overcoming the chilling requirement ranging from 4°C to 8°C (Dennis, 1987). Higher temperatures, above 18°C, will negate the chilling effect and temperatures lower than 2°C do not contribute towards the fulfilment of the chilling requirement (Couvillion and Erez, 1985). The response of seeds and

buds to chilling temperatures during dormancy tends to be similar (Dennis, 1996). In many species primary seed dormancy can be broken by exposure of seeds to low temperatures (stratification) (Lin *et al.*, 1979). However, information on seed and bud dormancy is based almost exclusively on a narrow genetic pool of species with high-chilling requirements (Fishman *et al.*, 1987). The mode of action of stratification treatments has been ascribed to the interaction between low temperatures and various germination promoters and inhibitors (Whitehead and Sutcliffe, 1995).

Exposure to low temperatures can break seed dormancy, stimulate seed germination and hence provide an insight into mechanism of seed dormancy. Therefore, the effect of plant growth regulators (GA_3, BA, kinetin, thiourea, jasmonic acid, zeatin) and their combinations on growth and development of stratified and non-stratified peach embryos as well as the ability of these substances to substitute for the need of low temperature exposure for germination were examined in this study.

Additionally, *in vitro* experiments were conducted to determine the effect of the following factors on embryo growth and development of three peach cultivars: the carbon source, protein source and the influence of gibberellic acid. Furthermore, the effect of varying the strength of the MS medium (quarter, half and full strength), the effect of addition of certain plant growth regulators (IBA, GA_3, and BAP) and of variation in photoperiod on growth and development of peach embryos were investigated.

4.2 Materials and Methods

4.2.1 Media preparation

The following media were evaluated for peach embryo culture: MS medium, (Murashige and Skoog, 1962), Woody Plant Medium (WP) (Lloyd and McCowan, 1981), Shives Medium

(Schenk and Hildebrandt, 1972) and Steward and Hsu Medium (SH) (1978). Additions to the media were carried out according to Rizzo *et al.* (1998) who established concentrations of 20 g l^{-1} sucrose, 200 mg l^{-1} casein and 0.01 mg l^{-1} GA_3 to be optimal for peach embryo development. The experimental layout is described in Appendix 8. The media were adjusted to pH 5.20 ± 0.02, using 1N KOH and 1N HCL (Kleber Chemicals Pty Ltd, Durban, South Africa). All media were mixed with agar (0.8 g l^{-1}) (Difco Bacto Agar, Sigma Chemical Co., St. Louis, USA) and autoclaved (HI-300 Autoclave, Huang Lin Medical Instruments Co. Ltd.,Taiwan) at 121°C for 30 min at 1.08 kPa, after which 10 ml of the medium were dispensed into a 65 mm diameter petridish. The effect of medium strength was determined on the cultivar 'Oom Sarel' using MS medium. This medium was supplemented with 0.01mg l^{-1} naphthalene acetic acid (NAA) (B. D. H. Laboratory Chemicals Division, Poole, England) and 0.2 mg l^{-1} 6-benzylaminopurine (BAP) (Sigma Chemical Company, St. Louis, USA.). The plant growth regulators were dissolved in 50 % ethanol (Merck Laboratory Suppliers Pty Ltd, Midrand, RSA).

Three peach cultivars were locally obtained: 'Summer Giant' (Mare's Silviculture, Greytown) an early ripening cultivar (October to November), 'Oom Sarel' (LA Rhone Trust, Tulbagh) a mid-season ripening cultivar (December to January) and 'Klara' (Maluti Producers, Ficksburg) a late-ripening cultivar (February to March). A total of 270 uniform fruits were used from each cultivar. The seeds were removed, sun dried and disinfected in a 1 % sodium hypochlorite solution (Mass Mart Holdings Pty Ltd, Sandton, RSA), after which the embryos were excised using a vice grip to break the stony endocarps. The embryos were then surface sterilized in 70 % ethanol and thereafter rinsed twice in ultrapure water.

4.2.2 Establishment of peach embryos in four different media with sucrose, casein and GA_3 as media additives

Embryos of the three above mentioned cultivars were cultured in 65 mm diameter petri dishes (Merck Pty Ltd., Midrand, RSA). Three embryos were placed into each petri dish and stratified at 1°C±2 for 40 days. The temperature regime and the stratification period were adopted from Perez-Gonzalez (1997). After stratification the petridishes were placed into germination chambers under conditions as described by Fouad *et al.* (1995) (25 °C/day and 20 °C/ night, 16 hour photoperiod for 30 days) (Appendix 8). Embryos were checked on a daily basis to determine the germination percentage. The experiment was terminated after 30 days, when most of rudimentary development has taken place. The percentage of normal plantlets, the number of roots and the plant fresh weight (fw) were recorded (embryos were considered germinated when the radicle has obtained the length of three mm or more). Data of days to germination were recorded as cumulative data for each medium, irrespective of sucrose, casein or GA_3 treatments. This procedure was adopted to determine the potential time period to 100 % germination in each of the different media.

4.2.3 Effect of MS medium strength and medium constituents on growth of embryos

Following the establishment of embryos in four different media, further experiments were carried out to determine the optimal conditions for further peach embryo development. MS medium which showed consistency and good results in previous experiments (4.2.2) was used together with different environmental culturing conditions. The procedure developed by Fouad *et al.* (1995) was used whereby 6-benzylaminopurine (BAP of 0.2 mg l^{-1}), gibberellic acid (GA_3 of 0.01mg l^{-1}), indolebutyric acid (IBA of 0.5 mg l^{-1}) and the combination of 0.5 mg l^{-1} IBA, 0.01mg

l^{-1} GA_3 and 0.2 mg l^{-1} BAP (Rizzo *et al.*, 1998) were tested as medium additives. The MS medium was prepared with the addition of 1 % agar and tested at full, half and quarter strength with and without addition of 20 g l^{-1} sucrose. Casein was not used in these experiments, it was replaced by use of plant growth regulators. The pH was adjusted to 5.2 as per Rahman *et al.* (1992). The cultures were incubated at 25°C/day and 20°C /night with a 16 hour photoperiod according to Rahman *et al.* (1992) (Appendix 9).

The effect of photoperiod was studied using half strength MS medium as this was found to perform well for tissues cultured *in vitro* (Rahman *et al.* 1992), supplemented with 0.1. mg l^{-1} NAA, 0.2 mg l^{-1} BAP and 20 g l^{-1} sucrose according to Rizzo *et al.* (1998). The pH was adjusted to 5.2 after which the medium was solidified by addition of 1g agar l^{-1} prior to autoclaving. Embryos were incubated at 24°C, 28°C and 32°C with 8, 12 and 16h day-length respectively according to Rahman *et al.* (1992).

Further evaluations of sucrose, agar concentrations, medium strength, as well as pH of the medium were carried out. The MS medium was supplemented with 0.1 mg l^{-1} NAA and 0.2 mg l^{-1} BAP. Concentrations of 0, 20, 40 and 60 g sucrose l^{-1} were tested in a medium containing 1.0 g agar l^{-1}. Furthermore ,pH levels of 4.2, 5.2, 6.2 or 7.2 were evaluated for determination of the optimal pH range for peach embryoculture. Agar concentrations of 0, 0.8, 1.5, 2.0 and 2.5 g l^{-1} were used to determine the optimal concentration, supplemented with 20 g l^{-1} sucrose and pH adjusted to 5.2. Each one of these factors (agar, sucrose and pH), was separately considered and evaluated with respect to growth and development of the embryos (Appendix 9).

4.2.4 Effect of stratification temperature and stratification period on dormancy release of peach embryos

Seeds of peach cultivars 'Oom Sarel', 'Summer Giant' and 'DeWet' with endocarp and without endocarp were either stratified or not stratified according to methods described by Frisby and Seeley (1993) and Seeley and Damavanandy (1985) (Appendix 10). The seeds were germinated in a greenhouse at a temperature of (25°C ±3) adopted from Seeley and Damavanandy (1985) and the germination percentage recorded after 15 days. A randomized complete block design was used with three replications and each treatment consisting of 30 seeds.

Furthermore, 60 seeds of 'Summer Giant' with protective stony endocarps and 60 seeds without endocarps (Frisby and Seeley, 1993), were planted in moist perlite, as described above, to evaluate the influence of temperature on the stratification of the embryos.

To determine the effect of plant growth regulators on stratification requirement of 'Oom Sarel' peach embryos, a further 270 peach seeds without the hard fruit endocarp were treated with various plant growth regulators as shown in (Appendix 10). In preliminary trials the concentration mentioned in Appendix 10 was found to be optimal for germination of peach embryos. Germination percentages obtained ranged from 75 % to 94 %. Peach embryos with endocarps removed were soaked for 24 hours (Chapparro and Moore, 1989) in the concentrations mentioned above without exposure to stratification temperatures. Thereafter they were transferred to the greenhouse for germination as described in 4.2.

4.3 Results

4.3.1 Evaluation of different media, various sucrose concentrations, addition of casein and GA_3 on *in vitro* growth of peach embryos

The media and plant growth regulators tested greatly influenced the development of peach embryos in all three peach cultivars studied. All the plant growth regulators tested influenced the germination percentage significantly (P=0.05), however there was no significant interaction between media and plant growth regulators in 'Klara' (Appendix 11). Embryos of all cultivars responded positively to the addition of sucrose, casein and gibberellic acid (Table 4.1).

There was no significant difference between the three cultivars tested with respect to growth and development of the embryos. However, 'Oom Sarel' obtained the highest germination percentage of 96.7 %, followed by 'Klara' with 93.3 % and 'Summer Giant' with 90.0 % (Table 4.1 a to 4.1 c) and germination percentage for all cultivars was approximately 60% (P=0.05). All four media were highly effective in sustaining embryo growth and development. MS and SH media showed the highest germination percentage of the three cultivars followed by WP and Shives (Table 4.1). MS medium produced best results for'Summer Giant' and 'Klara' embryos while SH medium was best for 'Oom Sarel' embryos (Table 4.1.).

Embryos were obtained by breaking the stony endocarp from the stony endocarp of 'Summer Giant' (Fig 4.1 A) and 'Oom Sarel' (Fig 4.1 B) were slow to react to the applied additives compared to 'Klara'. These embryos were characterized by slow germination, with the germination percentage increasing rapidly with time. 'Klara' (Fig.4.1. C) had higher germination percentage and displayed more rapid germination throughout the entire 30 days. It is, however, important to mention that embryos were able to germinate and develop during stratification period on all three cultivars tested, hence, germination percentage was recorded at day zero. Addition

of GA_3 (0.01 mg l^{-1}) resulted in the highest germination percentage (approximately 76.11 %) followed by casein (200 mg l^{-1}) (approximately 64.15 %) and sucrose (20 mg l^{-1}) (approximately 60.83 %) (Table 4.1).

Table 4.1

Influence of sucrose, gibberellic acid and casein on % germination of peach embryos (cv Summer Giant) cultured in four different media (MS, WP, Shives and SH)

4.1 a) 'Summer Giant'

	Media			
Media additives	MS	WP	Shives	SH
	% germination			
none (control)	50.0b	16.7b	26.7b	13.3c
sucrose	53.3b	33.3b	40.0b	36.7b
casein	73.3a	23.3b	63.3a	76.7a
GA_3	90.0a	90.0a	73.3a	86.7a
LSD (5%)	17.4			
SE (mean)	17.8			
CV %	24			

In any one column, means followed by the same letter(s) do not differ significantly from each other at P=0.05.

4.1 b) 'Oom Sarel'

	Media			
Media additives	MS	WP	Shives	SH
	% germination			
none (control)	20.0c	3.3c	20.0b	3.3c
sucrose	70.0b	26.7b	53.3a	83.3a
casein	20.0c	56.7a	53.3a	90.0a
GA_3	96.7a	60.0a	66.7a	36.7b
LSD (5%)	14.48			
SE (mean)	15.2			
CV %	18.8			

In any one column, means followed by the same letter(s) do not differ significantly from each other at P=0.05.

4.1 c 'Klara'

	Media			
Media additives	MS	WP	Shives	SH
	% germination			
none (control)	66.7b	60.0b	53.3b	43.3b
sucrose	93.3a	76.7a	86.7a	76.7a
casein	93.3a	73.3a	73.3a	73.3a
GA_3	90.0a	83.3a	76.7a	63.3a
LSD (5%)	20.01			
SE	21.3			
CV %	23			

In any one column, means followed by the same letter(s) do not differ significantly from each other at P=0.05.

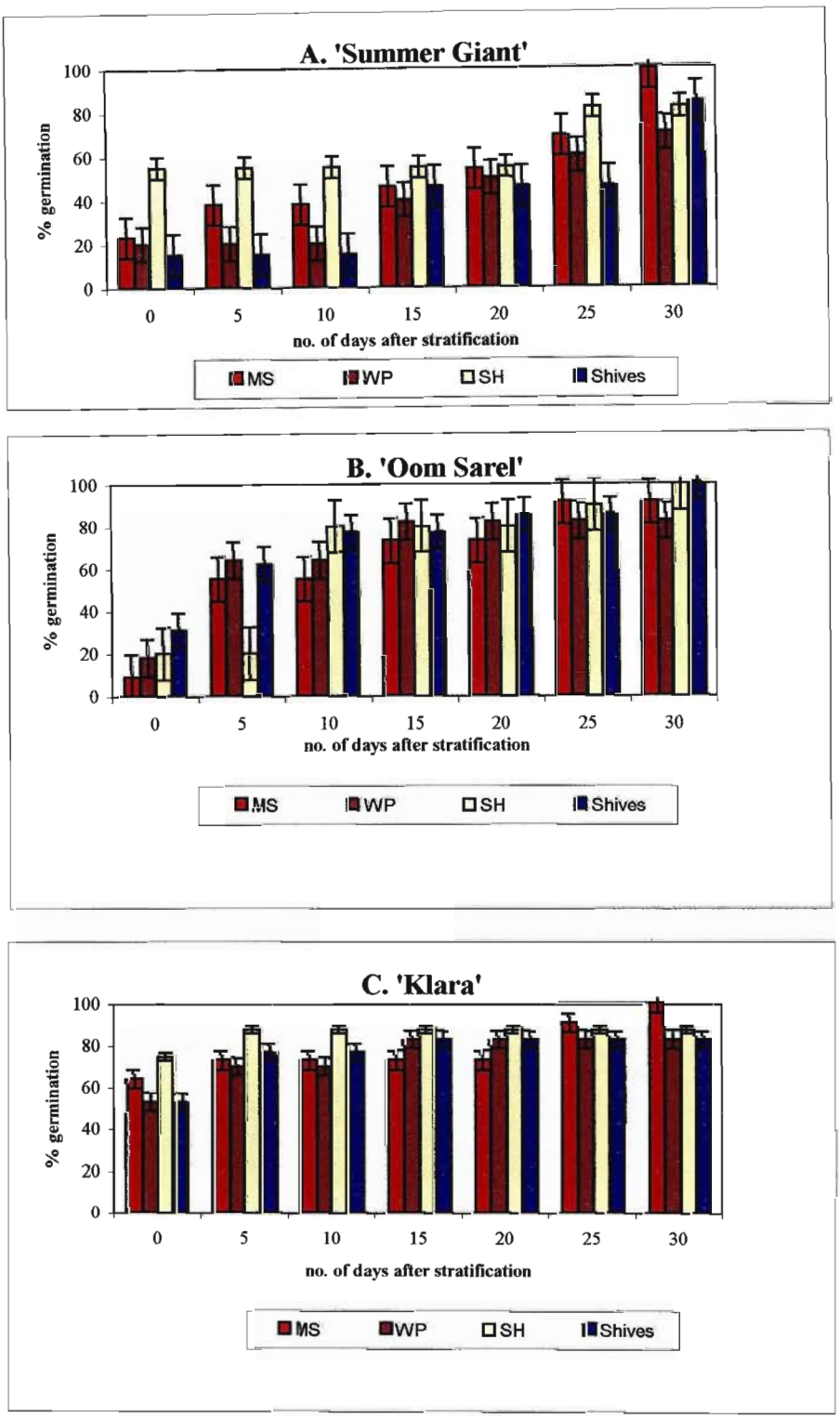


Fig 4.1: Germination percentage of stratified peach embryos, cultivars 'Summer Giant' (A), 'Oom Sarel' (B) and 'Klara' (C). Embryos were cultured in four different media (MS, WP, SH and Shives).

4.3.2 Effects of variations in MS medium composition on growth and development of peach embryos

4.3.2.1 Effect of MS medium strength

The strength of the MS medium as well as the addition of plant growth regulators to the medium influenced growth and development of embryos significantly (P=0.05). Incubated in the quarter strength medium, embryos showed generally good development with up to 93.3 % germination at 0.01mg l^{-1} GA_3, up to 8.7 roots per embryo and 6.7 cm average root length per embryo at 0.2 mg l^{-1} BAP + 0.5 mg l^{-1} $^{-1}$IBA + 0.01 mg l^{-1} GA_3 (Table 4.2). The half strength medium displayed even better results with different plant growth regulators. Embryos were able to obtain 100 % germination with both, 0.2 mg l^{-1} BAP and 0.2 mg l^{-1} BAP + 0.5 mg l^{-1} IBA + 0.01 mg l^{-1} GA_3 (Table 4.2). The full strength MS medium proved to be inhibitory to embryo development as it led to a reduction in the number of embryos forming roots (46.7 %), the mean number of roots per embryo (4.00) and the mean length of roots per embryo (2.53 cm) (Table 4.2).

4.3.2.2 Effect of photoperiod

Subjecting embryos to a light period of 16h light, resulted in the highest number of embryos germinating (96.70 %), higher number of roots per embryo (14.00) and higher mean root length per embryo (10.80 cm) than a 12h/12h or 8h/16h photoperiod at 24°C and 28°C incubation temperatures (Table 4.3). Peach embryos that were subjected to 12h light, 12h darkness showed significant embryo growth and development with varying incubation temperatures (Table 4.2). However, lower results compared to the other two photoperiods tested were shown by 8h day, 16h night photoperiod, (Table 4.3)

Table 4.2

Effects of MS strength and growth regulators (IBA, BAP, GA_3 and their combination) on embryo growth and development

In any one column, means followed by the same letter(s) do not differ significantly from each other at P=0.05.

strength of MS medium	plant growth regulator (mg l^{-1})	embryos germination (%)	mean no. of roots/embryo	mean root length/embryo (cm)
one quarter	BAP (0.2)	80.00b	7.00b	3.27b
	IBA (0.5)	43.30d	4.00c	5.93a
	GA_3 (0.01)	93.30a	8.00a	4.33b
	BAP (0.2) +IBA (0.5) + GA_3 (0.01)	83.30a	8.67a	6.73a
half	BAP (0.2)	100.00a	6.00b	7.57a
	IBA (0.5)	63.30c	6.00b	4.20d
	GA_3 (0.01)	93.30a	8.00a	5.20c
	BAP (0.2) +IBA (0.5) + GA_3 (0.01)	100.00a	8.00a	6.77b
full	BAP (0.2)	46.70d	2.670d	2.13f
	IBA (0.5)	33.30de	2.33d	2.20f
	GA_3 (0.01)	36.70d	4.00c	2.23f
	BAP (0.2) +IBA (0.5) + GA_3 (0.01)	23.00f	2.67d	2.53
LSD (5%)		10.88	1.2	0.88
SE (means)		12.91	1.42	1.04

Table 4.3

Effects of photoperiod and incubation temperature on root formation of embryos, the average number of roots per embryo and the average root length per embryo

In any one column, means followed by the same letter(s) do not differ significantly from each other at P=0.05.

Incubation temperature (°C)	photoperiod (Hours of light/dark period)	embryos germinated (%)	mean no. of roots/ embryo	mean root length / embryo (cm)
24	8/16	53.3f	3.00f	2.50c
	12/12	53.3f	4.00e	1.83c
	16/8	66.7d	5.00d	1.63c
28	8/16	80.0b	8.00c	7.13b
	12/12	83.3b	10.00b	7.12b
	16/8	96.7a	14.00a	10.80a
32	8/16	43.3f	1.67g	1.80d
	12/12	16.7g	1.33g	2.60c
	16/8	50f	2.00g	1.17d
LSD (5%)		12.05	0.642	1.344
SE (means)		12.7	1.361	1.357

4.3.2.3 Effect of sucrose, agar and pH

Lack of sucrose in the medium resulted in no evident growth and development of shoots or roots. Increasing the concentration from 0 to 20,40 and 60 sucrose g l^{-1} significantly increased the germination percentage, the mean number of roots per embryo and the mean length of roots per embryo. Best results were obtained at 60 g sucrose l^{-1} (Table 4.4). Without supply of agar to the medium, embryos turned brown and died, no development was recordable. Increasing the agar concentration to 1.5 g agar l^{-1} resulted in a higher germination than a lower or higher agar concentration. A concentration of 2.0 g agar l^{-1} gave the highest number of roots per embryo as well as longest root length but it the difference was not statistically significant to other

concentrations. The various agar additions were not significantly different from each other, only significantly higher when compared to the control (0 g agar l^{-1}) (Table 4.4).

A low pH (4.2) gave poor results in embryo development and growth. The pH of 5.2 resulted in significantly higher embryo germination mean number of roots per embryo and mean length of roots per embryo compared to other pH treatments (Table 4.4). The pH treatment was therefore, optimum at pH 5.2.

4.3.3 Effect of stratification temperature and duration on dormancy release of peach embryos

The length of the stratification period on the peach embryos was highly correlated with the number of days that the embryos spent at low temperatures (Table 4.8). The regression coefficients (R^2) ranged among cultivars from 0.8499 to 0.9971 in embryos 'with endocarp' and from 0.5833 to 0.9131 in embryos 'without endocarp' (Fig 4.3 to 4.5). Lower regression coefficients were found in embryos 'without endocarp' than in those 'with endocarp' between germination percentage and stratification period. This implies that not only the physical restrictions affect peach seed germination but also other factors that are inherent in the embryo such as plant inhibitors are involved. It is also important to note that in all three cultivars tested, the highest germination percentage was obtained after exposure to stratification period of 60 days (Fig 4.3 to Fig 4.5), whether the endocarps were removed or not. However, statistically there was no significant difference between 60 days and other stratification lengths. 'Oom Sarel' embryos were further tested under greenhouse conditions for determination of seedling growth parameters such as shoot growth, leaf area and root and shoot dry weight. These parameters displayed a strong linear relationship with the length of the stratification period, whether the embryos were removed from the endocarps or not (Fig 4.2).

Table 4.4
Effect of different sucrose, agar concentrations and varying pH values on root formation in embryos of peach cultivar 'Summer Giant'

In any one column, means followed by the same letter(s) do not differ significantly from each other at P=0.05.

medium additive	embryos germinated (%)	mean no. of roots/ embryo	mean root length/embryo (cm)
sucrose ($g\ l^{-1}$)			
0	0.0d	0.0c	0.0c
20	20.0c	3.3b	3.0b
40	50.0b	6.0b	4.1b
60	96.7a	12.7a	8.7a
LSD (5%)	7.64	3.48	2.12
± SE	14.38	1.85	1.13
agar ($g\ l^{-1}$)			
0	0	0.0b	0.0d
0.8	43.3c	3.1a	4.0b
1.5	93.3a	5.2a	7.0a
2	70.0b	5.5a	8.7a
2.5	13.3d	2.8a	3.0b
LSD (5%	21.05	4.45	3.41
± SE	11.11	2.36	1.81
pH			
4.2	23.3b	4.0b	2.2b
5.2	93.3a	12.0a	8.2a
6.2	36.7b	2.7b	3.3b
7.2	6.7b	1.0b	1.3b
LSD (5%)	30.26	3.44	3.01
± SE	16.07	1.81	1.63

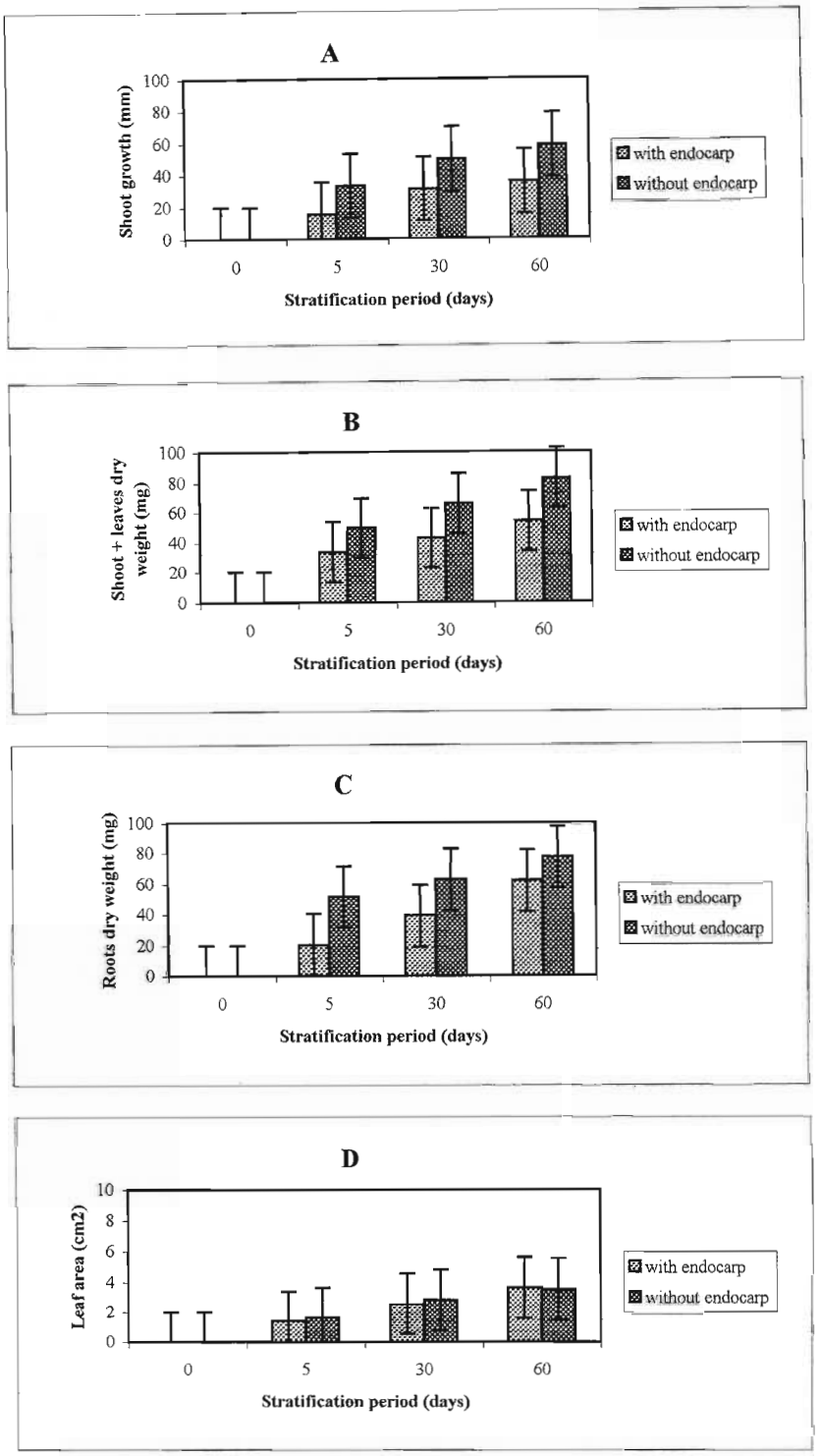


Fig 4.2: Effect of stratification period on growth and development of 'Oom Sarel' peach seeds with endorcarps and embryos removed from seeds. A (shoot growth), B (Shoot + leaves dry weight in mm), C (roots dry weight (mg) and D (Leaf area in cm^2)

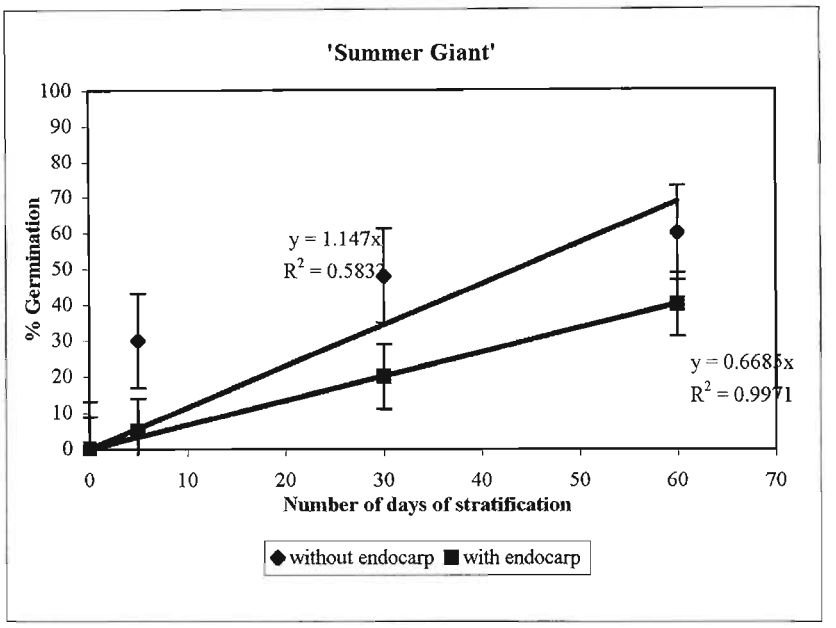


Fig. 4.3: Effect of stratification period on germination percentage of 'Summer Giant'. Standard errors tested at P=0.05

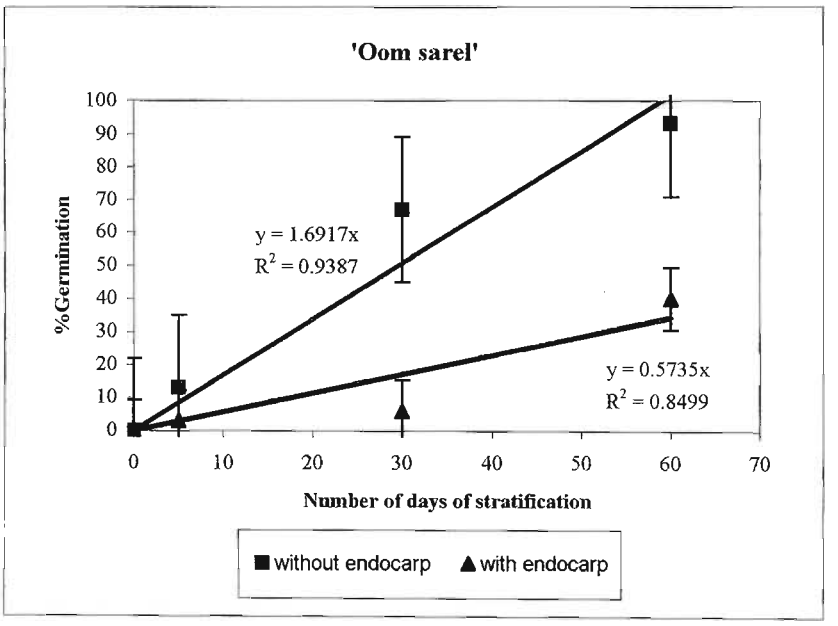


Fig. 4.4: Effect of stratification period on germination percentage of 'Oom Sarel'. Standard errors tested at P=0.05

Embryo development was greatly influenced by temperature. A strong regression coefficient of R^2=0.945 was found between seeds without endocarps and incubation temperature, while seeds with endocarp and temperature showed a regression coefficient of R^2=0.1305 (Fig.4.6). At room temperature (22± 2°C) none of the embryos germinated, but decreasing the temperatures to 16°C, 8°C and 4°C increased the germination percentage significantly. Seeds that were stratified at 4°C (without endocarp),obtained the highest germination percentage (90%).

All plant growth regulators tested in this study increased germination percentage of non-stratified peach embryos (Table 4.5). Incubation in 0.01 mg l^{-1}. Gibberellic acid resulted in significantly higher germination percentage than incubation in other plant growth regulators. BA and JA significantly promoted germination in peach embryos, obtaining 53 % and 67 % germination respectively. However, the combination between BA and JA obtained best results when compared to other combinations and important to note is the fact that the combination between either JA or BA with GA_3 became inhibitory in promoting germination.

Table 4.5
Influence of plant growth regulators on development of non-stratified embryos

In any one column, means followed by the same letter(s) do not differ significantly from each other at P=0.05.

Plant Growth Regulators	% Germination	% Survival
Control	10.0c	19.0c
GA_3 (0.1mg l^{-1})	73.0a	60.0b
Kinetin (150 mg l^{-1})	46.0b	76.0b
BA (50 mg l^{-1})	53.0b	70.0b
Thiourea (4 g l^{-1})	44.0b	62.0b
Jasmonic acid (0.1mg l^{-1})	67.0a	64.0b
GA_3 (0.1mg.l^{-1}):BA (50 mg l^{-1})	39.0b	68.0b
JA (0.1mg l^{-1}):GA_3 (0.1 mg l^{-1})	38.0b	89.0a
BA (50 mg l^{-1}):JA (0.1 mg l^{-1})	89.0a	82.0a
overall mean	51	65.6
LSD (at 5%)	17.5	16.7

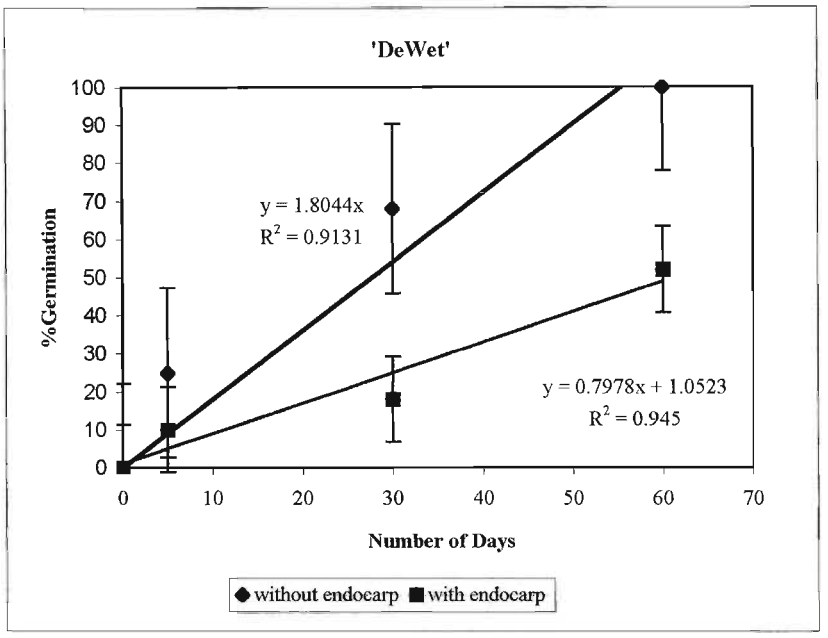


Fig 4.5: Effect of stratification period on germination percentage of 'DeWet' embryos. Standard errors tested at P=0.05

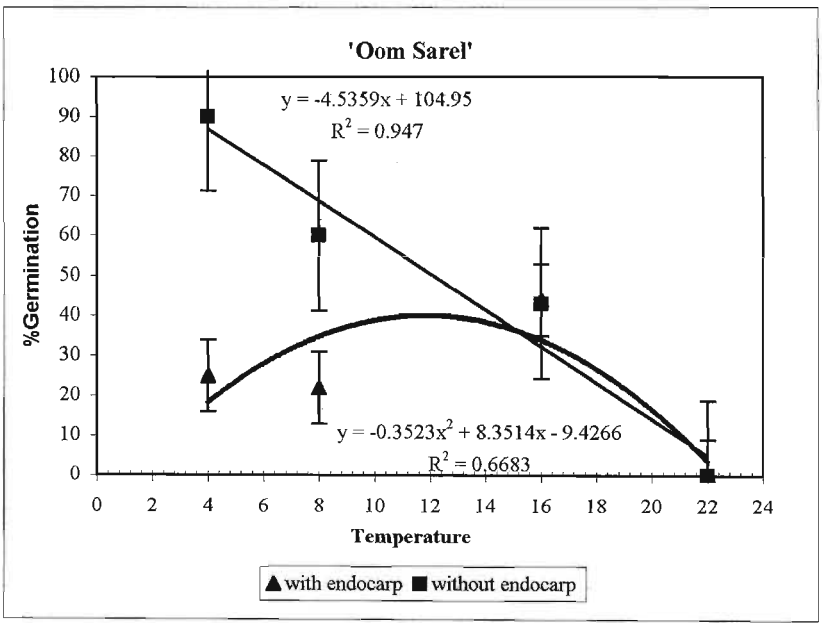


Fig. 4.6: Influence of stratification temperature on germination percentage of 'Oom Sarel' embryos Standard errors tested at P=0.05

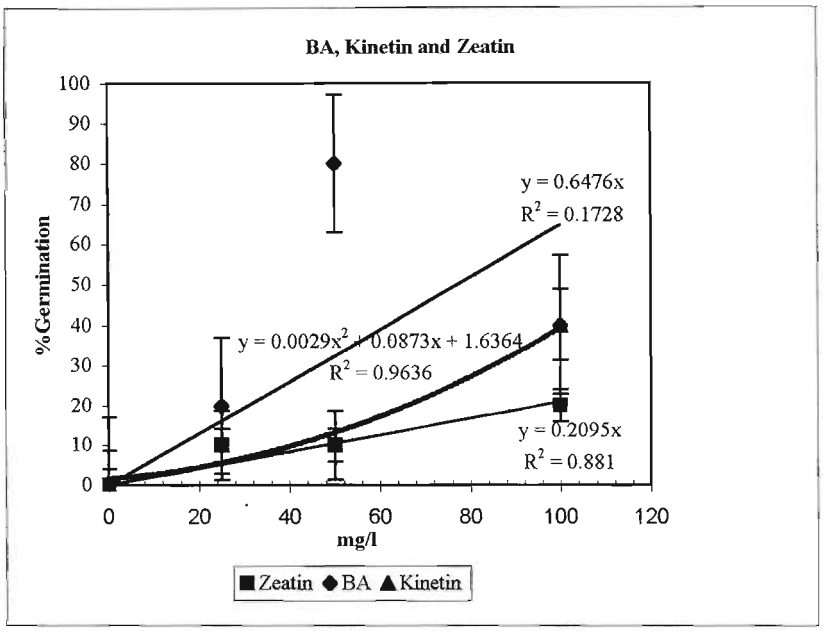


Fig. 4.7: **Effect of BA, Kinetin and Zeatin on germination percentage of non-stratified peach embryos. Standard errors were tested at P=0.05**

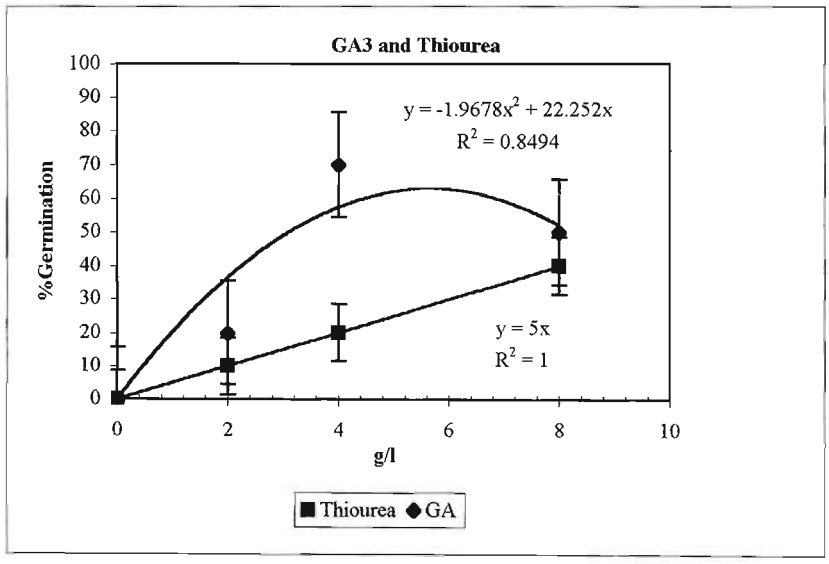


Fig.4.8: **Effect of Thiourea and Gibberelic acid on germination percentage of non-stratified peach embryos. Standard errors were tested at P=0.05**

4.4 Discussion

The composition of the media significantly affected the germination percentage of excised peach embryo of all three cultivars tested. The high levels of ammonium salts in MS and SH media (1.6 g/100 ml $NH_4 NO_3$ and 3.0 g/100 ml $NH_4 NO_3$ respectively) might have been responsible for the increased germination of peach embryos, as these forms of nitrogen provide a readily available source for the growing embryos (George, 1993). The cultivar Klara had the highest percentage germination at 30 days. 'Klara' is a late ripening cultivar, it is possible however, that at harvesting the embryos of this cultivar were fully mature and just need after-ripening period that will be enough to break dormancy (Antonelli *et al.*, 1988). Cultivars 'Summer Giant' and 'Oom Sarel' also obtained good germination percentages but growth and development was slower as embryos from early-ripening cultivars often are underdeveloped at fruit ripening (Antonelli *et al.*, 1988).

The addition of casein, sucrose and GA_3 to the four media tested affected embryo germination, mean number of roots per embryo and mean length of roots per embryo. Casein addition at 200 mg l^{-1} in four different media tested resulted in significant growth and development. However, the role of casein in germination and overall plantlet growth is not understood (Rizzo *et al.*, 1998). The promotive effect of gibberelic acid on shoot growth and the enhancement of root development confirms earlier results (Scozzoli *et al.* 1991). These authors obtained improved shoot and plantlet development resulting from application of GA_3 on peach embryos cultured *in vitro*. It is, however, likely that a further increase of the GA_3 concentration (from 0.01 mg l^{-1} to 0.02 mg l^{-1}) could have resulted in better growth and development of the embryos. Sucrose can be used as a carbohydrate source for in vitro embryo development. It is believed that addition of the medium provided the most needed carbon source for embryo development and subsequent growth.

The addition of three growth regulators GA_3, BAP and IBA to the culture medium gave the highest germination (100%), number of roots per embryo (8.0) and root length per embryo (7.57 cm) when supplied to a half strength MS medium. The promotive effect of plant growth regulators on induction of shoot formation has been found to be dependent on the level of macronutrients and micronutrients in the medium (Raghavan, 1994). It is, however, believed that these plant growth regulators are important in the promotion of and maintenance of germination, especially GA_3 (Bewly, 1997). Gibberellins are known to obviate the requirement of seeds for various environmental cues, promote germination and counteract the inhibitory effects of ABA, whilst cytokinins are important in radicle and shoot development (Bewley, 1997). Strengths of the MS medium tested varied significantly over different plant growth regulators used. It was observed, that half strength resulted in better embryo development and growth than both quarter strength and full strength. This observation supports the results obtained by Rahman et al. (1992), who reported that half MS medium gave highest rooting in Rosa explants, cultivar Tajmahal.

Photoperiod, temperature, pH and agar concentration of the medium are believed to function together with plant growth regulators in promoting shoot development in tissue culture (George, 1993). In this study the 16h light/8h dark period with a temperature of 28 ° C gave the highest rooting percentage of embryos (96.7 %). Increasing the temperature to 32 ° C was inhibitory to embryo growth and development and lower temperature (24 ° C) did not significantly influence embryo development. Hence, peach embryo development is better at relatively high temperatures, several degrees above normal soil temperatures in the culture medium (15 ° C to 23 ° C) (Antonelli et al.,1988). These results are in line with earlier reports by Lane (1978), who showed that apples shoots cultured *in vitro* at 28 ° C day/22 ° C night had a survival rate of 94 %. A reduction in the culture temperatures to 23 °C / 17 °C or 18 °C / 12 ° C resulted in a progressive

reduction in number of roots formed.

The sucrose concentration of the medium, its pH and agar concentration significantly influenced the development and establishment of peach embryos cultured *in vitro*. Development was found to be progressing faster at sucrose concentration of 60 g/l, 1.5 g l^{-1} agar concentration and pH 5.2. These results support findings reported by Smith and Spomer (1995), that higher concentrations of sucrose (40 to 80 g sucrose l^{-1}) in a medium, solidified with 1.0 to 2.5 g agar l^{-1}, at the pH ranges of 4.2 to 6.5 give best root and shoot development in most plants cultured *in vitro*. A further increase in sucrose concentration from 60 g l^{-1} to 80 g l^{-1} might have resulted in even faster embryo development.

Removal of the endocarps resulted in earlier germination in all three cultivars studied (Fig 4.3 to Fig 4.5). 'De Wet' gave 100 % germination when the embryos were separated from the stony endocarp (Fig 4.5) compared to 60 % in seeds where the endocarps were left intact. Chang *et al.* (1984) found that in peach seeds a delay in seed germination may be associated with the presence of an inhibitor. Furthermore, results obtained by Carlson and Tukey (1945) showed that peach seeds contain inhibitors which are located mainly in the seed coat and endocarp and that the removal of the endocarp will ultimately lead to shorter time for seeds to germinate and achieve 100% germination in a shorter time period compared to seeds where the endocarps were not removed.

However, the studies undertaken have shown that poor germination of peach embryos may not be limited by underdeveloped embryos at fruit maturity only, but other factors such as after-ripening period and chilling requirement are necessary in temperate deciduous fruit species. Exposure to cold temperature for various time periods (2 ° C to 8 ° C) induced a germination

percentage of up to 90 % in all three cultivars tested. However, at high temperatures (22 ° C), total germination of 'Oom Sarel' was low (Fig 4.6) (60 %), demonstrating the need for chilling of peach seeds to increase germination. Therefore temperate fruit species, such as peach, need to be exposed to low temperature before satisfactory germination can be obtained. Perez-Gonzalez (1990) mentioned the existence of a close relationship between the chilling requirement to promote germination in peach seeds and the increased germination percentage of these seeds. These results also support findings of Perez-Gonzalez (1990) that stratification of peach seeds at 5 to 7 ° C increase germination up to 90 % in less than 55 days.

All plant growth regulators tested in this study proved to be important in stimulating germination of non-stratified peach embryos (Fig 4.7 and Fig 4.8). The significant results obtained from application of 0.01 mg l^{-1} GA_3 (73 % germination), 50 mg l^{-1} IBA (53 % germination) and 0.01 mg l^{-1} JA (67 % germination) (Table 4.5) clearly demonstrated the role played by these growth regulators in substituting the chilling requirement and enhancing germination in non-stratified peach embryos. Pinfield and Davies (1978), showed that the mode of action of cytokinins in seed germination is to increase the sensitivity of seeds to gibberellin and/or ethylene. Gibberellins have also been shown to stimulate germination and substitute for the low temperature requirement of certain seeds for germination (Derkx and Karssen, 1994). The role of the plant hormone jasmonic acid has been shown in seed dormancy (Martin-Closas, 2000) and in particular in stimulating potato plantlet development (Ravnikar, *et al.*, 1992) and in particular in stimulating potato plantlet development (Zimerman and Vick, 1983). Gibberellic acid has been reported to accumulate in peach seeds during the pit hardening stage and at seed maturity (Vithaya, 1990). It is possible that an increase in seed germination from 10 % to 73 % of non-stratified seeds treated with GA_3 stimulated the endogenous GA_3 levels which is necessary for germination.

Exogenous application of growth regulators has been shown to cause metabolic changes in seeds and in turn induce germination (Mehanna *et al.*, 1985). Biochemical studies have shown that plant growth regulators modulate transcription and translation, resulting in an induction of quantitative and qualitative changes in protein synthesis in seeds (Naidu *et al.*, 2000). Noland and Murty (1984) found that growth regulators triggered germination of the embryos through activation of specific enzymes which hydrolyse storage molecules (such as carbohydrates) and catalyse essential reactions in energy generating cycles during seed germination (to provide energy and structural components for the growth and emergence of the embryos). A similar mechanism of action might have occurred in peach cultivars studied.

The possibility that the removal of the stony endocarps (which impose para-dormancy) resulted in better germination percentage than in seeds where the endocarps were not removed is viewed as the major break-through in peaches like in other species (Hartmann *et al.*, 1997) on which future studies can be based. Furthermore the involvement of plant growth regulators in increasing germination of non-stratified peach embryos from 10 % to 89 % demonstrates the important involvement of these growth regulators to counteract the activity of germination inhibitors within the embryo. It seems likely that both embryo and endocarp impose dormancy onto peach seeds implying a possible combination of para-dormancy (a specific biochemical signal originating in a structure other than the affected structure as the initial reaction) (Amen, 1968), and endo-dormancy (the phenomenon on where the initial reaction leading to control of growth is a specific perception of an environmental or endogenous signal within (i.e., endo) the affected structure alone (e.g. embryo).

CHAPTER FIVE

Conclusions and Future Prospects

This study has demonstrated that propagation of peaches using cuttings and seeds can be used as a practise to produce future rootstocks for orchard establishment. Further, it was found that both IBA treatments and rooting-cofactors studied increased rooting percentage in peach cuttings. This finding makes propagation of peaches using cuttings extremely useful in areas that are still experiencing poor plantlets establishment. The study also showed that a strong and highly complex relationship between peach seed dormancy and use of plant growth regulators exist. This dormancy can be broken by use of plant growth regulators.

Seedling rootstock production by embryoculture seems to be under-exploited means of peach production. Of several media tested MS medium, half strength proved to be most suitable for *in vitro* culture of peach embryos. Addition of casein to the medium increased growth and development of embryos. The addition of other amino acids preparations and potentially their combinations, might further improve growth and development of peach embryos. Enriching the medium with other plant growth regulators and varying the concentrations of the already tested plant growth regulator combinations (especially those containing GA_3), may also improve growth and development of *in vitro* peach embryos. Embryos developed *in vitro* normally grow into uniform seedlings, which can serve as selection material of new rootstocks in nurseries. Rootstocks developed from embryos are able to grow fast under optimum conditions of embryoculture, display uniform growth and can be used as future rootstocks. Therefore, peach embryos can be grown *in vitro*, hardened in a greenhouse, forced, grafted and be tested as rootstock-scion combination at less than one year of age and show high compatibility and

resistence.

Peach rootstocks are used as rootstocks for plum (*Prunus domestica*), apricot (*Prunus armeniaca*), cherry (*Prunus pumila*) and for peach (*Prunus persica*) itself. It is therefore important to search for rootstocks with a wider amplitude of resistance to drought, cold, root-knot nematodes and other environmental obstacles and to produce rootstocks with a high compatibility towards scions of several *Prunus* species.

Future studies should also focus on improving the survival rate of embryos raised *in vitro* when used as rootstock or in rootstock-scion combinations after being planted out in the orchard. These performance tests should be carried through to full production. This includes the effect of such rootstocks on precocity, yield, fruit quality and disease tolerance as well as resistance to adverse environmental conditions.

Further anatomical studies should focus on elucidating the involvement of the cells of the cambial region in ARF, as light microscopy results have revealed that cells of the cortex and cells within the vicinity of the cambial region are involved in ARF. These findings will set the basis through which future auxin applications can be manipulated and applied in cuttings.

Outcomes of this research will therefore benefit minor peach production areas, such as Lesotho, which are characterized by poor germination of seeds used for seedling rootstock production (especially in nurseries, producing rootstocks), poor plant establishment and lack of uniform orchards. Findings obtained in this study can be adopted in an attempt to optimise peach production and high-light the possibilities of successfully commercializing this fruit in such areas. It is therefore concluded that factors limiting peach production in Lesotho such as pest and

diseases, drought, low yield/unit area and poor fruit quality can me minimized by proper orchard establishment practices. Such practices would include among others, proper rootstock establishment and selection. Development of proper plantlets either from seeds, embryos and cuttings will yield high quality rootstocks, that can be used to establish proper orchards and reduce time taken from establishment to tree fruit bearing age as well as improving the fruit quality and ultimately the yield.

Appendices

Appendix 1: Influence of IBA on rooting of peach cuttings; Experimental layout

A. 'DeWet'

Experimental unit	Treatment (IBA concentration (mg l^{-1})	Position of cutting
		Terminal
1	0 (control)	
2	500	
3	1000	
4	2000	
5	0 (control)	Basal
6	500	
7	1000	
8	2000	

B. 'Florida Prince'

Experimental unit	Treatment (IBA concentration (mg l^{-1})	Position of cutting
		Terminal
1	0 (control)	
2	500	
3	1000	
4	2000	
5	0 (control)	Basal
6	500	
7	1000	
8	2000	

C. 'Earlibelle'

Experimental unit	Treatments (IBA concentration (mg l^{-1})	Position of cutting
		Terminal
1	0 (control)	
2	500	
3	1000	
4	2000	
5	0 (control)	Basal
6	500	
7	1000	
8	2000	

Appendix 2: Influence of IBA+ rooting-cofactors on rooting of peach cuttings; experimental layout

A. 'DeWet'

Experimental unit	Position of cutting	Treatment factor (IBA concentration (mg l^{-1}) + rooting cofactor (100 µg l^{-1})
1	Terminal	0 IBA + phloroglucinol
2		500 IBA + phloroglucinol
3		1000 IBA + phloroglucinol
4		2000 IBA + phloroglucinol
5	Basal	0 IBA + phloroglucinol
6		500 IBA + phloroglucinol
7		1000 IBA + phloroglucinol
8		2000 IBA + phloroglucinol

B. 'Florida Prince"

Experimental unit	Position of cutting	Treatment factor (IBA concentration (mg l^{-1}) + rooting cofactor (100 µg l^{-1})
1	Terminal	0 IBA + phloroglucinol
2		500 IBA + phloroglucinol
3		1000 IBA + phloroglucinol
4		2000 IBA + phloroglucinol
5	Basal	0 IBA + phloroglucinol
6		500 IBA + phloroglucinol
7		1000 IBA + phloroglucinol
8		2000 IBA + phloroglucinol

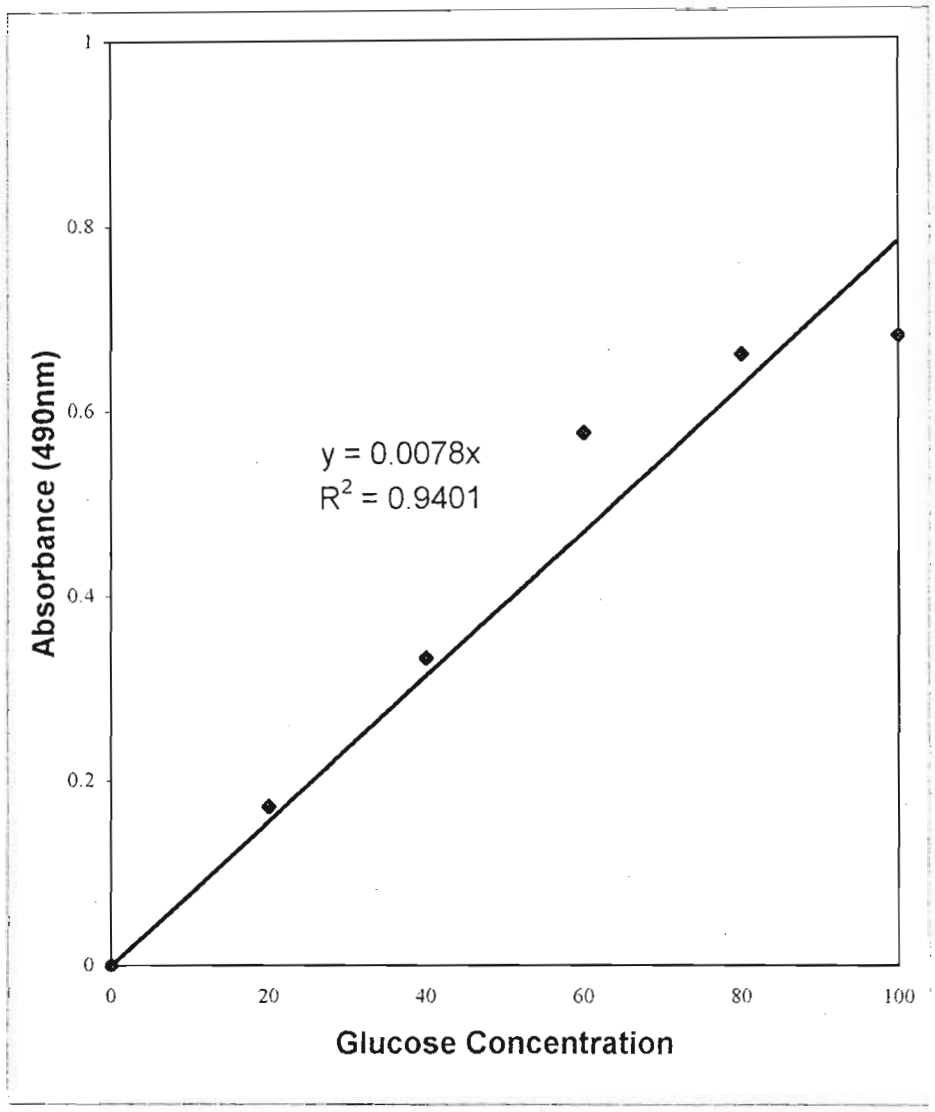
C. ' Earlibelle'

Experimental unit	Position of cutting	Treatment factor (IBA concentration (mg l^{-1}) + rooting cofactor (100 µg l^{-1})
1	Terminal	0 IBA + phloroglucinol
2		500 IBA + phloroglucinol
3		1000 IBA + phloroglucinol
4		2000 IBA + phloroglucinol
5	Basal	0 IBA + phloroglucinol
6		500 IBA + phloroglucinol
7		1000 IBA + phloroglucinol
8		2000 IBA + phloroglucinol

Appendix 3: Experimental layout for the influence of IBA and Ca-EDTA on rooting of "wounded" and "non-wounded" peach hardwood cuttings of 'Florida Prince'

Experimental unit	Treatment factor
1	0 mg l^{-1} IBA (no wounding)
2	1000 mg l^{-1} IBA
3	100 mg l^{-1} CA-EDTA
4	induced wounding
5	1000 mg l^{-1} IBA + induced wounding
6	1000 mg l^{-1} IBA + 100 mg l^{-1} Ca-EDTA + induced wounding
7	1000 mg l^{-1} IBA + 100 mg l^{-1} Ca-EDTA + non-induced wounding
8	100 mg l^{-1} Ca-EDTA + induced wounding

Appendix 4: Standard curve used for the determination of carbohydrate content in plant tissues (Glucose concentration in $\mu g\ l^{-1}$)



Appendix 5: Statistical analysis (Anova)

Effects of rooting-cofactors, IBA, cultivar and cutting position on rooting of peach cuttings

Source of variation	Degrees of freedom	Mean sum of squares	F. probability
RC	3	5562	0.001
IBA	3	2308.3	0.001
CV	2	2389.4	0.004
PC	1	82.3	0.657
RC IBA	9	873.1	0.032
RC CV PC	6	189.2	0.842
IBA CV	6	749.3	0.101
RC PC	3	230.9	0.646
IBA PC	3	100	0.868
CV PC	2	163.1	0.677
RC IBA CV	18	692.2	0.049
RC IBA PC	9	425.5	0.425
RC CV PC	6	219.7	0.787
RC IBA CV PC	18	393.4	0.465
Residual	192	498.5	0.267
Total	287		

Each treatment consisted of 10 cuttings and was replicated three times. A factorial design (in a randomized complete block design) was used. Data were analysed using Anova programme Genstat 5 (4.1 Release, 4th Edition, Lawes Agricultural Trust IACR-Rothamsted)

Abbreviations used in the table:

RC - Rooting-cofactors CV- Cultivar

IBA- Indolebutyric acid PC- Cutting position

RC IBA- Combination of rooting-cofactors and indolebutyric acid.

RC CV PC- Combination of rooting-cofactors, cultivars and position of the cutting.

IBA. CV- Combination of indolebutyric acid and cultivars.

RC PC- Combination of rooting-cofactors and the position of the cutting.

IBA. PC- Combination of indolebutyric acid and the position of the cutting.

CV PC- Combination of cultivars and the position of the cutting.

RC IBA CV- Combination of rooting-cofactors, indolebutyric acid and cultivars.

RC IBA PC- Combination of rooting-cofactors, indolebutyric acid and the position of the cutting.

RC CV PC- Combination of rooting-cofactors, cultivars and the position of the cutting.

RC IBA CV PC- Combination of rooting-cofactors, indolebutyric acid, cultivars and the position of the cutting.

Appendix 6:

Analysis of variance showing difference in rooting (%), number of roots/cutting and length of the root as influenced by IBA, Ca-EDTA and wounding (Table shows significant differences in eight treatments tested)

Source of variation	Degrees of freedom	Mean sum of squares			F probability
		Rooting (%)	Average number of roots/cutting	Average length of roots/cutting	
Treatment (IBA,Ca-EDTA or Wounding)	7	2261.9	44.841	54.857	0.01
Residual	16	162.5	4.730	7.833	
Total	23				

Each treatment consisted of 10 cuttings and was replicated three times. A complete randomized design (CRD) was used. Data were analysed using Anova progamme GENSTAT 5 (4.1 Release, 4th Edition, Lawes Agricultural Trust IACR-Rothamsted)

Appendix 7:

Dehydration series (a) wax embedding (b) and the staining procedure (c) used for sample preparation for light microscopy studies.

(a) Dehydration series

step	v: v: v: ratio of water: ethanol: butanol	minimum time (h) in solution	temperature (°C) of solution
1	45: 45: 10	1	20
2	30: 50: 50	12	20
3	15: 50: 35	1	20
4	15: 40: 55	1	20
5	0: 25: 75	1	20
6	0: 0: 100	2	40
7	0: 0: 100	18	40

Chemicals supplied by Merk Laboratory suppliers Pty Ltd, Johannesburg, South Africa

b) Wax embedding Series

solution	minimum time (h) in solution	temperature (°C) of solution
Butanol : Liquid paraffin (3:1 v/v)	24	40
Liquid paraffin (100%)	12	40
Liquid paraffin + wax pellets (1:1 v/v)	12	40
Liquid paraffin + wax pellets (1:3 v/v)	24	60
Pure molten wax	48	60

Chemicals supplied by Merk Laboratory suppliers Pty Ltd, Johannesburg, South Africa

c) staining procedure

step	solution	time
1	Xylene (100%)	10 min
2	Xylene (100%)	10 min
3	Ethanol : Xylene (7:3 v/v)	5 min
4	Ethanol (100%)	10 min
5	Ethanol (80%)	5 min
6	Ethanol (70%)	5 min
7	Safranin (100%)	2 h
8	Distilled water	30 s
9	Picric acid : Ethanol (1:1v/v)	5 s
10	NH_3 solution (%) : Ethanol (1:1 v/v)	2 min
11	Ethanol (100%)	5 min
12	Fast Green : Clove oil (1:1 v/v)	30 s
13	Clove oil (100%)	30 s
14	Clove oil : Xylene : Ethanol (1: 1: 1 v/v)	5 s
15	Xylene (100%)	10 min.

Chemicals supplied by Merk Laboratory suppliers Pty Ltd, Johannesburg, South Africa

Appendix 8:

Experimental layout for the determination of the effect of culture medium and its constituents. Concentrations of the supplements were according to Rahman *et al.* (1992) and Rizzo *et al.* (1998)

Culture medium	Medium supplement	pH	Agar concentration
MS	a) 20 g l^{-1} sucrose b) 200 mg l^{-1} casein c) 0.01 mg l^{-1} GA_3 d) no supplement	5.2	0.8 g l^{-1}
WP	a) 20 g l^{-1} sucrose b) 200 mg l^{-1} casein c) 0.01 mg l^{-1} GA_3 d) no supplement	5.2	0.8 g l^{-1}
Shives	a) 20 g l^{-1} sucrose b) 200 mg l^{-1} casein c) 0.01 mg l^{-1} GA_3 d) no supplement	5.2	0.8 g l^{-1}
SH	a) 20 g l^{-1} sucrose b) 200 mg l^{-1} casein c) 0.01 mg l^{-1} GA_3 d) no supplement	5.2	0.8 g l^{-1}

Appendix 9:

Experimental layout for studying the effect of culturing conditions on peach embryo development according to methods of Rahman *et al.* (1992) and Rizzo *et al.* (1998).

Factor under evaluation	Plant growth regulators	Medium strength	pH	Temperature	Photoperiod	Sucrose concentration	Agar concentration
plant growth regulators	0.2 mg l^{-1} BAP or 0.01 mg l^{-1} GA_3 or 0.5 mg l^{-1} IBA or 0.2 mg l^{-1} BAP + 0.01 mg l^{-1} GA_3 + 0.5 mg l^{-1} IBA	quarter half full	5.2	25°C/day and 20°C/night	16 h photoperiod	20 g l^{-1}	1 g l^{-1}
Photoperiod	0.1 mg l^{-1} NAA or 0.2 mg l^{-1} BAP	half	5.2	a) 24°C b) 28°C c) 32°C	8, 12, and 16h photoperiod	20 g l^{-1}	1 g l^{-1}
sucrose concentration	0.1 mg l^{-1} NAA or 0.2 mg l^{-1} BAP	half	5.2	24°C	16h photoperiod	a) 0 g l^{-1} or b) 20 g l^{-1} or c) 40 g l^{-1} or d) 60 g l^{-1}	1 g l^{-1}
pH	0.1 mg l^{-1} NAA or 0.2 mg l^{-1} BAP	half	4.2 or 5.2 or 6.2 or 7.2	24°C	16h photoperiod	20 g l^{-1}	1 g l^{-1}
agar concentration	0.1 mg l^{-1} NAA or 0.2 mg l^{-1} BAP	half	5.2	24°C	16h photoperiod	20 g l^{-1}	0 g l^{-1} or 0.8 g l^{-1} or 1.5 g l^{-1} or 2.0 g l^{-1} or 2.5 g l^{-1}

Appendix 10:

Experimental layout for stratification temperature and duration

Stratification period (days)	Seeds with endocarps	Seeds with endocarps removed	Cultivar used
0	4°C ±1	4°C ±1	'Oom Sarel' 'Summer Giant' 'DeWet'
5			
30			
60			
Stratification temperature			
Room temperature	stratified for 30 days	stratified for 30 days	'Summer Giant'
4 °C			
8 °C			
16 °C			
Plant growth regulators			
GA_3 (0.0 1mg l^{-1})	none	not stratified, soaked for 24 hours in the growth regulators	'Oom Sarel'
Kinetin (150 mg l^{-1})			
BA (50 mg l^{-1})			
Thiourea (4 g l^{-1})			
Zeatin (150 mg l^{-1})			
JA (0.1 mg l^{-1})			
GA_3 (0.01 mg l^{-1}) +BA (50 mg l^{-1})			
BA (50 mg l^{-1}) + JA (0.1 mg l^{-1})			
JA (0.1 mg l^{-1}) + GA_3 (0.01mg l^{-1})			

Appendix 11:

Anova tables showing influence of sucrose, gibberellic acid and casein with four media on growth and development of peach embryos

a) 'Summer Giant'.

TREATMENTS	Degrees of freedom	Mean sum of squares	F probability
MEDIA	3	2616.7	0.001
PGR'S	5	6773.3	0.001
MEDIA*PGR'S	15	415.6	0.001
RESIDUAL	48	112.5	
TOTAL	71		

Each treatment consisted of 10 cuttings and was replicated three times. A randomized complete block design was used. Data were analysed using Anova programme Genstat 5 (4.1 Release, 4th Edition, Lawes Agricultural Trust IACR-Rothamsted)

b) 'Klara'.

TREATMENTS	Degrees of freedom	Mean sum of squares	F probability
MEDIA	3	1508.8	0.001
PGR'S	5	2142.5	0.001
MEDIA*PGR'S	15	54.4	0.982
RESIDUAL	48	148.6	
TOTAL	71		

Each treatment consisted of 10 cuttings and was replicated three times. A randomized complete block design was used. Data were analysed using Anova programme Genstat 5 (4.1 Release, 4th Edition, Lawes Agricultural Trust IACR-Rothamsted)

c) 'Oom Sarel'

TREATMENTS	Degrees of freedom	Mean sum of squares	F probability
MEDIA	3	859.26	0.001
PGR`S	5	8342.22	0.001
MEDIA*PGR`S	15	1210.37	0.001
RESIDUAL	48	77.78	
TOTAL	71		

Each treatment consisted of 10 cuttings and was replicated three times. A randomized complete block design was used. Data were analysed using Anova programme Genstat 5 (4.1 Release, 4th Edition, Lawes Agricultural Trust IACR-Rothamsted).

Appendix 12:

Anova table of plant growth regulators and germination percentage and survival percentage.

	% Germination			% Survival	
Sources of variation	Degrees of freedom	Mean sum of squares	F. probability	Mean sum of squares	F probability
PGR`s	8	5320	0.002	3960	0.008
Residual	81	1531		1364	
Total	89				

Each treatment consisted of 10 cuttings and was replicated three times. A complete randomized (CRD) design was used. Data were analysed using Anova programme Genstat 5 (4.1 Release, 4th Edition, Lawes Agricultural Trust IACR-Rothamsted)

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