IN VITRO CULTURE OF THE PASSION FRUIT, PASSIFLORA EDULIS SIMS

NELSON ONZERE AMUGUNE

A Thesis submitted in partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN BOTANY

UNIVERSITY OF NAIROBI

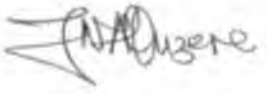
APRIL 1992

DEDICATION

This thesis is dedicated to my wife Beatrice and son Andrew as well to the entire family of Mr. and Mrs. Eliakim Onzere

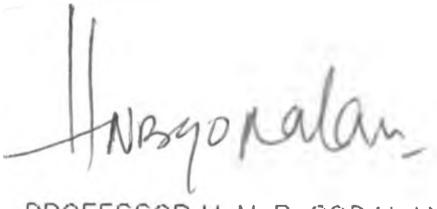
DECLARATION

This Thesis is my original work and has not been submitted for award of a degree in any other University

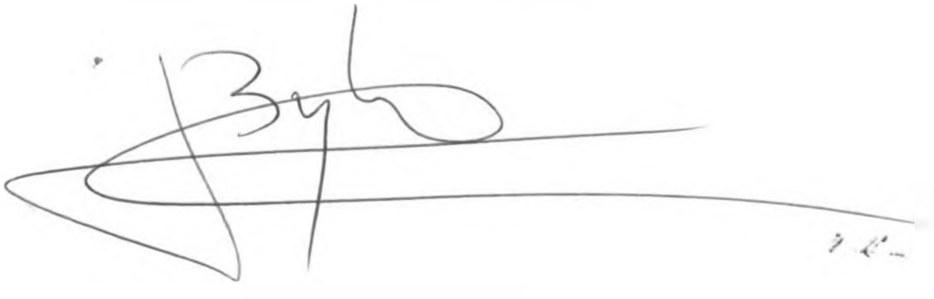


NELSON ONZERE AMUGUNE

This Thesis has been submitted with our approval as University Supervisors



PROFESSOR H. N. B. GOPALAN



MR. BENNY BYTEBIER

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisors, Professor H. N. B. Gopalan and Mr. Benny Bytebier of the Department of Botany, University of Nairobi, for taking keen a interest in this study and for their invaluable guidance which made this study successful.

I would like to thank Professor T. K. Muklama and Dr. D. Van Speybroeck of the Botany Department, who introduced me to certain concepts that helped me through the study. My thanks also go to Mr. F. Njeru and Mr. H. Okang'a of the Botany Department, and to Miss K. W. Njoroge, for their technical assistance. I thank Mr. L. M. Kigondu of the Zoology Department, for his assistance in photography.

I would like to thank the following individuals from the Kenya Agricultural Research Institute (KARI); Mr. Njue of the Mtwapa Research Centre and Mr. Kanyagia of the National Horticultural Research Centre, Thika, for kindly supplying us with passion fruit seeds and plants.

I would also like to thank Professor J.-P. Hernalsteens, of the Free University Brussels, Belgium for kindly supplying us with the Agrobacterium strains used in the study.

Furthermore, I would like to thank the VVOB (the Flemish Association for Development Cooperation and Technical Support) for supplying us with some of the materials and for use of their computer equipment.

Lastly, I am indebted to Hoechst (E. A.) for providing me with free samples of the antibiotic Claforan® (cefotaxime) which proved very useful in the transformation experiments.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

In Vitro Culture of the Passion Fruit, Passiflora edulis Sims

In this study, a tissue culture method, which can be used for multiplication of the yellow passion fruit is described. Callus was initiated from different explants of the yellow and purple passion fruit. Immature seeds of var. edulis formed callus when inoculated on MS (Murashige and Skoog) medium containing 1-3 mg/l α-napthaleneacetic acid (NAA) and 0.5 mg/l 6-benzylaminopurine (BAP). Stem explants of var. edulis and var. flavicarpa formed callus when inoculated on MS media supplemented with 2 mg/l NAA, with or without 0.5 mg/l BAP. Stem segments with nodes formed shoots when inoculated on MS medium with 2 mg/l BAP or 2 mg/l BAP and 0.2 mg/l NAA.

Leaf discs of var. flavicarpa formed callus when inoculated on MS medium supplemented with 2 mg/l NAA, 2 mg/l indole-3-acetic acid (IAA) and 0.4 mg/l kinetin (KIN). Those inoculated on medium with 2 mg/l NAA or 2 mg/l NAA and 0.5 mg/l KIN formed callus and roots within 4 weeks. Shoots were observed on leaf discs of var. flavicarpa inoculated on MS medium supplemented with 2 mg/l BAP or 2 mg/l BAP and 0.5 mg/l KIN within 4 weeks. The shoots were then subcultured onto MS medium supplemented with 0.1 mg/l BAP for further growth. Such shoots could be induced to root when subcultured onto MS media containing 0.1, 0.5 and 1 mg/l NAA. Rooted plantlets could be obtained within 8 weeks.

In this study, investigations were also carried out to determine whether passion fruit is a host for infection by Agrobacterium.

Agrobacterium tumefaciens is a Gram-negative soil bacterium that can cause the formation of tumours on certain wounded plants. This bacterium harbours an oncogenic Ti plasmid which enables it to induce crown gall tumours on most dicotyledonous and some monocotyledonous plants. The Ti plasmid has a segment, called the T-DNA, which is transferred from the bacterium and becomes stably integrated into the plant genome. Hence, A. tumefaciens can be used as a vector to transfer foreign genes into plants.

Results obtained showed that passion fruit is susceptible to Agrobacterium infection. Tumours were induced on the plant infected with the hypervirulent A. tumefaciens strain A281. These tumours could grow on MS medium without hormones, free of the inciting bacteria. Agropine, a compound whose synthesis is directed by A. tumefaciens genes, could be detected in the tumour tissue. These findings open the possibility of improving passion fruit by genetic engineering.

Experiments done to investigate the problem of seed dormancy in passion fruit confirmed that the tough seed coat could be the major cause. It was established that dormancy could be broken by scarification and subsequent treatment of the scarified seeds with gibberellic acid.

CHAPTER ONE

Introduction and Literature Review

1.1 Passion fruit

Passion fruit belongs to the family Passifloraceae genus Passiflora. This family comprises 12 genera and 500 woody and herbaceous species (Kuhne, 1968; Bailey, 1973). The genus Passiflora includes more than 350 species of sub-tropical vines, which climb by tendrils. Only a few are grown for their edible fruit (Kuhne, 1968), these include P. edulis. P. ligularis, P. quadrangularis, P. mollissima, P. antioquiensis, P. laurifolia and P. foetida (Purseglove, 1968). Of these species, P. edulis and P. quadrangularis are the most commonly cultivated species throughout the tropics. P. caerulea is grown as an ornamental (Bailey, 1973).

1.1.1 Origin and distribution

Passiflora edulis is native to southern Brazil. It was introduced into England in 1810, Hawaii in 1880, to Tanzania from Natal, South Africa in 1896 (Purseglove, 1968; Akamine et al., 1974). Commercial production of the fruit in Kenya started in 1935 (Nattrass, 1939; Chapman, 1963; Samson, 1986). Other countries that grow passion fruit on a commercial scale include Australia, Fiji, Sri Lanka, Brazil, New Zealand, South Africa, Malawi and Zimbabwe (Kuhne, 1968; Purseglove, 1968; Akamine et al., 1974; Samson, 1986).

1.1.2 Cultivars

Two cultivars of P. edulis are recognised: P. edulis var. edulis is the purple passion fruit while P. edulis var. flavicarpa is the yellow passion fruit. Both cultivars are grown in Kenya. Fruits of P. edulis var. edulis are 4-5 cm in diameter and deep purple when ripe. P. edulis var. flavicarpa has lager fruits, 5-6 cm in diameter, which are deep canary yellow when ripe. The pulp of var. flavicarpa is rather more acid. P. edulis var. flavicarpa is of uncertain origin. Degener (1933) reported that it is a form of P. edulis which arose by mutation. This idea has been disputed by Pope (1935), who claims that it may have arisen in Australia as a hybrid of P. edulis with a closely related species. This variety is better suited to

the tropical lowlands while var. edulis does best at higher altitudes of about 1800 m (Purseglove, 1968).

In Brazil, var. flavicarpa is the predominant type grown even though the purple form (var. edulis) can also be found (Maluf et al., 1989). In Australia, Sri Lanka and India, however, var. edulis is most exploited (Pruthi, 1963). Both the yellow and the purple forms are grown in Australia, but the former is generally used as a gratting stock for the latter (Cox and Kiely, 1961). Both rain-loving and drought-tolerant forms of var. edulis are grown in Brazil (Martin and Nakasone, 1970).

1.1.3 Ecology

Purple passion fruit needs a moist climate with at least 1000 mm rain (Samson, 1986). In Kenya, this variety is grown in highlands, at an altitude of 1000-1800 m. The yellow form on the other hand, is better suited to the tropical lowlands, from sea level to about 750 m. This variety is normally grown in the Coast Province of Kenya.

1.1.4 Properties

The properties of var. edulis and var. flavicarpa are compared in table 1. Purple passion fruit has very desirable fruits. However, it has a lower yield than the yellow form and lacks resistance to diseases and pests. The yellow form, though having resistance to diseases and giving a higher yield, has a higher acid content.

1.1.5 Seed germination or breaking of dormancy

Some dormant seeds require a specific environmental stimulus which triggers germination. Factors that can cause seed dormancy include having a hard seed coat that is impermeable to water or gases, or is physically resistant to embryo expansion, having a specific light requirement, or harbouring the presence of a substance that inhibits germination (Delvin and Witham, 1983). A hard seed coat is one of the most common factors associated with seed dormancy.

Table 1: Some properties of two varieties of P. edulis (After Samson. 1986)

Variety	var. edulis	var. flavicarpa
Plant vigour	medium	high
Yield (tonnes/ha/yr)	8-10	10-20
Juice content	medium	high
Acidity	medium	high
Tolerance to cold	yes	no
Tolerance to nematodes	no	yes
Tolerance to woodiness	no	yes
Tolerance to Fusarium spp.	no	yes
Altitude range (in m)	1000-1800	0-750

Germination can sometimes be promoted by scarification of the seeds by mechanical or physical means. Scarification refers to any method that renders the seed coat permeable to water and oxygen or breaks the seed coat so that embryo expansion is not physically retarded. Mechanical scarification is effected by any treatment of the seeds that will crack or scratch the seed coats, such as shaking the seeds with some abrasive material (e.g. sand) or scratching or nicking the seed coat with a knife (Delvin and Witham, 1983).

Chemical scarification is also an effective way of breaking dormancy resulting from the seed coat. Other methods for breaking this type of dormancy are: dipping seeds into strong acids, such as sulphuric acid, or into organic solvents, such as acetone or alcohol, and then rinsing with water. Potassium nitrate stimulates germination of certain dormant seeds (Palevitch and Thomas, 1976). Even boiling water may be a successful treatment (Dell, 1980). As in mechanical scarification, chemical scarification breaks dormancy by weakening the seed coat or by dissolving the waxy material that renders the seed coat impervious to water. Hormones are considered to be the primary germinating agents. The stimulatory effect of applied gibberellic acids on the germination of both dormant and non-dormant seeds has been widely reported (Villiers, 1972). Gibberellic acids will stimulate germination in seeds where dormancy or quiescence is imposed by a wide variety of mechanisms, e.g. incomplete embryo development, mechanically resistant seed coats, presence of germination inhibitors, and factors relating to the physiological competence of the embryo axis (Jones and Stoddart, 1977).

Passion fruit seeds are usually prepared by washing them in water to remove the pulp and then dried either in the sun or at room temperature. Such seeds, when sown may take up to 8 weeks to germinate and have a germination rate of 85% after storing for 3 months (Purseglove, 1968). For faster germination, the seeds are usually soaked in water for 48 hours before sowing. This reduces the period required by the seeds to germinate to 3-5 weeks. Seeds may also be planted from ripe fruits without removal from the pulp and will then germinate in 2-4 weeks (Purseglove, 1968). Apart from treatment with water, there are no

reports describing other treatments that can break seed dormancy in passion fruit, hence the need for further work to be done.

1.1.6 Propagation

Passion fruit can be propagated by seed, cuttings or grafts. In Kenya, and in many other countries where passion fruit is cultivated, commercial plantings rely on seedlings (Lippman, 1978). Seeds are readily available as they are extracted from the pulp during processing. The main disadvantage of using such seeds is the inability to distinguish between seeds from high quality fruits and those from lower quality fruits.

In Kenya, for example, high quality fruits are used for export since they fetch higher prices and those of lower quality are used for processing. Ironically, many farmers obtain their seeds from the factories which process these fruits. A programme of selecting seeds from high quality fruits, like the ones exported, is lacking in Kenya.

In other countries, vegetative propagation is becoming increasingly important in order to obtain disease-resistant plants and clonal material of known parentage. This vegetative propagation is carried out mainly from rooted cuttings and grafting (Gilmour, 1983).

1.1.7 Uses

The fruits have several uses: the pulp and seeds may be eaten directly from the shell or used in fruit salads. The yellow gelatinous aromatic pulp around the seeds is used for making jams, jellies, and ice cream. The most important use, however, is in making juice which is used as a beverage and for making wine (Purseglove, 1968; Samson, 1986). The fruits are good sources of vitamin A and niacin (Martin and Nakasone, 1970).

1.1.8 Improvement

For juice production selection is based on flavour, thinness of rind, well filled cavity and yield of juice. Colour and content of sugar, acid and vitamins may also be taken into consideration. Resistance to woodiness disease and brown spot is also required. With the yellow passion fruit in Hawaii it was found that oval fruits contain about 10% more juice than the round fruits (Purseglove, 1968). The improvement of the purple passion fruit undoubtedly

awaits more thorough collection and distribution of wild type forms and selection of adapted varieties (Martin and Nakasone, 1970). Although, some improved strains of var. flavicarpa are available, they usually do not fruit well in plantings of single clones, or without adequate insect pollinators (Akamine and Girolami, 1959; Nishida, 1963; Knight and Winters, 1963).

1.1.9 Diseases and pests

Passion fruit pests include the Kenya mealy-bug, giant coreid bug, leaf-footed plant bug, stink bug, aphids, yellow mites, systates weevil and nematodes. The major diseases of the fruit are the brown spot, a disease induced by the fungus Alternaria passiflorae and woodiness caused by passion fruit woodiness virus (Ministry of Agriculture, 1981). Whereas the pests and brown spot disease can be controlled by pesticides and other chemicals there is no known chemical control for woodiness. The affected plants are usually burnt (Ministry of Agriculture, 1981).

One of the ways of solving the problem of woodiness is to obtain initially virus free cuttings of var. edulis and graft them on seedlings of var. flavicarpa. This is particularly important since var. flavicarpa is resistant to woodiness. Another advantage of var. flavicarpa is its resistance to nematodes and soil-borne fungi. In Kenya, however, there is no grafting programme between the two varieties going on at present. The problems of woodiness, nematodes and soil-borne fungi therefore still persist in the purple variety.

To overcome the problem of nematodes and soil-borne fungi, farmers have been advised to rotate their crops. For every hectare of passion fruit, two hectares must be available for rotation (Samson, 1986). This practice has caused many farmers to discontinue growing the crop especially in Kisii district due to pressure on land. However, a host-parasite study is now being undertaken to investigate the relationship between the nematodes and the purple variety (Kanyagia, personal communication).

Of the diseases mentioned, the most serious problem of passion fruit in Kenya is woodiness disease (Nattrass, 1939; 1940; Purseglove, 1968). This disease which is thought to be transmitted by aphids causes the fruits to be gradually reduced in size. It also causes the

rind to become hard and little pulp is produced leading to severe losses. Among the pests, root nematode is the most important and its best remedy is rotation (Samson, 1986). Efforts to obtain improved varieties should lay a lot of emphasis on obtaining disease resistant plants, especially against woodiness disease as suggested by Nattrass (1938).

1.1.10 Processing and marketing

Passion fruits are marketed either as fresh fruits or as processed products. There are two main factories in Kenya that process the fruits. One is situated at Thika and the other one is at Machakos. The market for Kenya's products is wide and consists of both the local and export market. The price for fresh purple fruits used in processing is about K.Sh. 5.00 per kg (September, 1991). For fresh fruits exported, the farmers earn K.Sh. 8.00 to K.Sh. 12.00 per kg.

Table 2 shows the export figures for fresh purple passion fruit from 1985 until 1991, while table 3 compares the 1991 export figure of passion fruit with that of other horticultural crops.

The main problem facing the export market is quality. Kenya is facing stiff competition from other countries like Brazil, Australia, Zimbabwe and Malawi which apparently produce higher quality fruits. Kenya, therefore, has to produce improved varieties that can yield high quality fruits if it is to compete favourably with other passion fruit-growing countries.

Hybridisation between the purple and the yellow passion fruits appears to be useful in transferring some of the desirable characteristics from one variety to the other (Martin and Nakasone, 1970). While P. edulis var. edulis is considered to have superior flavour, P. edulis var. flavicarpa is resistant to Fusarium and yields more abundantly. However, plants originated from a Hawaiian hybrid of purple x yellow fruit were slightly higher yielding than the Brazilian yellow passion fruit (Maluf et al., 1989). In Kenya, such a cross has also been made between the purple form (female) and the yellow form (male) and a fertile F1 hybrid has been obtained (Njue, personal communication). The hybrid has not yet been characterised.

Table 2: Export figures for fresh purple passion fruit

(Ref: Horticultural Crops Development Authority- 1992)

Year	Quantity exported (in tonnes)
1985	421.40
1986	645.73
1987	334.68
1988	337.52
1989	343.03
1990	445.83
1991	619.11

Table 3: 1991 export figures for some horticultural crops

(Ref: Horticultural Crops Development Authority- 1992)

Crop	Quantity (in tonnes)	Income (in K. Sh.)
Passion fruit	619.11	21,049,706
Mango	1,745.17	56,369,088
Avocado	4,193.03	62,895,420
French bean	14,854.96	516,952,538
Paw paw	73.96	1,035,440
Flowers	16,405.01	984,300,720

Apart from this F1 hybrid, there is no other programme, to our knowledge, going on in Kenya, that aims at obtaining improved passion fruit varieties.

1.2 Plant Tissue Culture

One possible way of obtaining improved varieties of passion fruit is by development of a plant tissue culture system for the crop. The term plant tissue culture is a convenient term to describe all types of sterile plant cell culture procedures pertaining to the growth of plant cells, tissues, organs, embryos, protoplasts and plantlets (Tisserat, 1985). It is an important method in the study of plant metabolism, plant genetics, plant morphogenesis and plant physiology. Genetic transformation of plants, elimination of plant pathogens, preservation of important plant species or varieties in limited space and rapid multiplication of plants can also be achieved through plant tissue culture (Murashige, 1974; Reinhert and Bajaj, 1977; George and Sherrington, 1984; Leifert and Waites, 1990).

A reliable *in vitro* method for the propagation of passion fruit would have considerable benefits for the horticulture industry by allowing the rapid clonal propagation of superior-yielding, disease-resistant selections as well as allowing clonal multiplication of superior rootstock material in areas where grafted passion fruit plants are required (Kantharajah and Dodd, 1990).

1.2.1 Callus cultures

A callus consists of an amorphous mass of loosely arranged parenchyma cells arising from the proliferating cells of the parent tissue (Dodds and Roberts, 1982). Callus cultures may be derived from a wide variety of plant organs, for example, roots, stems, shoots, leaves or more specialised explants such as pollen and endosperm (Dixon, 1985). The most important characteristic of callus is that this abnormal growth has the potential to develop normal roots, shoots, and embryoids which form plantlets (Dodds and Roberts, 1982).

Callus is usually formed at the cut end of the stem or root frequently as a result of wounding (Dodds and Roberts, 1982; Dixon, 1985). The stimuli involved in the production of wound callus are the endogenous hormones auxin and cytokinin. Callus may be initiated by

auxin alone, cytokinin alone, auxin and cytokinin, and by complex natural products (Yeoman and Macleod, 1977; Dodds and Roberts, 1982). Callus proliferation usually occurs in the dark since light tends to promote embryogenesis, shooting and greening of the callus (Dixon, 1985). Young tissues are more suitable than mature ones for callus initiation especially in the case of leaves. Callus may appear yellowish, white, green or pigmented with anthocyanin (Dodds and Roberts, 1982).

For callus induction, explants are transferred and gently pressed into the semi-solid medium. Stem sections may produce more callus if planted vertically with one cut end in the agar (Dixon, 1985). After a callus has been grown for a period of time in association with the original tissue, it becomes necessary to subculture the callus to a fresh medium. Continuous growth on the same medium leads to depletion of essential nutrients and a gradual desiccation of the agar because of water loss. Metabolites secreted by the growing callus may also accumulate to toxic levels in the medium (Dodds and Roberts, 1982).

During subculture, callus is usually cut into smaller pieces (5-10 mm) in diameter and weighing 20-100 mg and transferred to fresh medium (Street, 1969). If the inoculum is too small, it may exhibit very slow growth or none at all. Yeoman and Macleod (1977) suggest that cultures maintained on agar at 25°C or above should be subcultured every 4-6 weeks.

1.2.2 Plant regeneration

Plant regeneration through tissue culture can be accomplished using one of the three methods: embryo culture, somatic embryogenesis and organogenesis. (Krikorian, 1982; Tisserat, 1985). Plant development through organogenesis is the formation and outgrowth of shoots from callus or initiation and outgrowth of axillary buds generated from cultured tips and their subsequent adventitious rooting (Tisserat, 1985).

Plant production through organogenesis can be achieved in three main ways: by production of adventitious organs from a callus derived from the explant, by production of plantlets from outgrowth of axillary buds and by emergence of adventitious organs directly from the explant without an intervening callus phase (Tisserat, 1985).

Roots, shoots and flowers are the organs that may arise de novo from plant tissue cultures. Skoog and Miller (1957) observed that a relatively high concentration of auxin : cytokinin ratio induced root formation in tobacco callus, whereas a low ratio of the same hormones favoured shoot production. Whereas shoot initiation or caulogenesis in cultured plant tissues can be induced in many systems by an appropriate balance of exogenous auxin and cytokinin, in some cases, either one or the other of these growth regulators must be omitted from the medium in order to produce buds (Street, 1977; Dodds and Roberts, 1982). In contrast, monocot cultures may not need exogenous cytokinin to initiate buds (Street, 1977).

Sometimes, supplements or omissions of auxin : cytokinin have failed to induce shoots in the culture of many species. Street (1977) suggested that this failure could be due to the fact that additional hormones may be required. For example, gibberellic acid induces shoots in Chrysanthemum callus. Endogenous hormones may also accumulate and inhibit organogenesis, an effect that is not reversed by exogenous hormones. Cultural conditions involving nutritional and physiological factors may also block the on set of organogenesis (Street, 1977; Dodds and Roberts, 1982).

For leaf disc regeneration most of the success has been achieved from plants in the family Solanacae such as Nicotiana tabacum, N. sylvestris, Solanum nigrum, Petunia hybrida, Atropa belladona and Lycopersicon esculentum (for a review: see Tisserat, 1985). Leaf disc regeneration has also been realised in Begonia hiemalis (Begoniaceae), Brassica olaracea (Cruciferae), Perrilla frutescens (Labiatae), Coffea arabica (Rubiaceae), Prunus amayadalis (Rosaceae) and Vitis spp. (Vitaceae) (Tisserat, 1985).

In the family Passifloraceae, leaf disc regeneration has been realised in only one species: Passiflora alato-caerulea (Muralidhar and Mehta, 1982; 1986). To our knowledge, reports describing a leaf disc regeneration system for P. edulis var. edulis and P. edulis var. flavicarpa are lacking.

1.2.3 Tissue culture in Passiflora

The following tissue culture systems for various species of Passiflora have been described (Nakayama, 1966; Montaldi, 1972; Moran Robles, 1978; Scorza and Janick, 1979; 1980; Muralidhar and Mehta, 1982; 1986; Desai and Mehta, 1985; Kantharajah and Dodd, 1990).

Nakayama (1966) obtained callus and adventitious shoots from stem explants of P. caerulea. Similar experiments were carried out by Muralidhar and Mehta (1982) on P. alato-caerulea using shoot apices, stem internodes, pre-chilled axillary buds and leaf discs. Shoots were induced from shoot apices (2-3 mm) of P. alato-caerulea on Murashige and Skoog (MS) medium (1962) supplemented with 0.1 mg/l α-napthaleneacetic acid (NAA) and 0.5 mg/l 6-benzylaminopurine (BAP). Stem internodes (10-12 mm.) formed shoots on MS medium supplemented with 0.2 mg/l NAA and 2 mg/l BAP, while leaf discs formed shoots on the same medium supplemented with 2 mg/l indole-3-acetic acid (IAA), 2 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) and 2 mg/l BAP. Pre-chilled axillary buds formed shoots on MS medium supplemented with 2 mg/l BAP and 0.02 mg/l kinetin (KIN). Further shoot growth was realised by subculturing the shoots onto MS medium supplemented with 0.2 mg/l NAA and 0.5 mg/l BAP. The shoots induced in all these cases were induced to root on MS medium supplemented with 0.5 mg/l indole-3-butyric acid (IBA)(Muralidhar and Mehta, 1982).

Further experiments showed that direct shoots could be initiated from leaf discs of P. alato-caerulea on MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BAP (Muralidhar and Mehta, 1986). The growth of the shoots would then be enhanced by subculturing to MS medium supplemented with 0.2 mg/l NAA and 0.5 mg/l BAP. Rooting was induced by subculturing the shoots onto MS medium supplemented with 0.5 mg/l IBA (Muralidhar and Mehta, 1986).

Scorza and Janick (1979) initiated callus from leaf discs of P. caerulea using MS medium supplemented with 1 mg/l NAA and 1 mg/l BAP. The callus formed shoot initials after

subculturing onto MS medium supplemented with 1 mg/l NAA. Desai and Mehta (1985) induced shoots from leaf discs of P. alato-caerulea on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l KIN. Callus and roots were induced from leaf discs inoculated on MS medium supplemented with 2 mg/l NAA and 0.5 mg/l KIN, while those on MS medium supplemented with 2 mg/l IAA, 2 mg/l NAA and 0.4 mg/l KIN, formed callus only (Desai and Mehta, 1985). Montaldi (1972) induced buds on roots of entire plants of P. caerulea using liquid Hoagland and Arnon (1938) medium supplemented with 1 mg/l KIN.

Leaf discs of P. edulis var. flavicarpa formed callus when inoculated on MS medium supplemented with 1 mg/l NAA and 1 mg/l BAP. However, no regeneration was observed (Scorza and Janick, 1979). Moran Robles (1978) induced shoots via basal callus from axillary buds of P. edulis var. flavicarpa on MS medium supplemented with 2 mg/l KIN. Plantlet regeneration was obtained when the shoots were subcultured onto MS medium supplemented with 2 mg/l IAA or 2 mg/l 2,4-D or both.

Leaf discs of P. foetida callused when inoculated on MS medium supplemented with 1 mg/l NAA and 1 mg/l BAP. Only rhizogenesis was observed after subculturing the callus to MS medium with 1 mg/l BAP (Scorza and Janick, 1979).

Axillary buds of P. mollissima formed shoots via basal callus when cultured on MS medium supplemented with 2 mg/l KIN. Plantlets were obtained after subculturing the callus to MS medium supplemented with 2 mg/l KIN with or without 2 mg/l IAA (Moran Robles, 1978).

Leaf discs of P. suberosa formed shoots and flowers after being cultured on MS medium supplemented with 1 mg/l NAA and 1 mg/l BAP (Scorza and Janick, 1979). Further experiments by the same authors showed that in vitro flowering could also be induced from stem and tendril explants of P. suberosa on MS medium supplemented with 0.1 mg/l BAP (Scorza and Janick, 1980).

Kantharajah and Dodd (1990) described a technique for the micropropagation of P. edulis from seedlings explants. The seedlings were obtained after germinating the seeds on

MS medium, in approximately 25 days. Shoot multiplication from nodal segments of the seedlings was realised using MS medium supplemented with 2 mg/l BAP with or without coconut water. To avoid chlorosis, subculturing was done every 4 weeks when the shoots were transferred to MS medium without hormones for 1-2 weeks before subculturing them either on a further multiplication medium or onto a root induction medium. Roots were initiated on shoots that were 10-15 mm long, using MS medium supplemented with 1 mg/l NAA. No roots were observed when 1 or 2 mg/l IBA was used.

Similar experiments using MS medium supplemented with 3 mg/l IAA and 0.05 mg/l KIN produced considerable callus from the nodal segments but very few shoots. When leaf discs were used, callus and root initials were induced at the edge of the middle vein on MS medium supplemented with 0.5 mg/l NAA and 1.5 mg/l BAP within 4 weeks, but no shoots were observed (Kantharajah and Dodd, 1990).

In summary, reports describing tissue culture systems for var. edulis and var. flavicarpa starting from leaf discs are still incomplete as only rhizogenesis has been realised but no shoots have been obtained, hence the need for further work.

1.3 Somaclonal variation

Plants regenerated from somatic cells using tissue culture are not always genetically uniform but may display significant genetic variability. This variability has been termed as somaclonal variation (Evans and Sharp, 1986; Karp, 1989) and can be used to select clones with new traits like disease resistance, increased yield and herbicide resistance.

Somaclonal variation may also be used to generate variants of a commercial cultivar in high frequency without hybridising with other genotypes (Larkin and Scowcroft, 1981). Single gene and organelle gene mutations have been recovered as a result of somaclonal variation. In Lycopersicon esculentum, for example, single gene mutations were recovered from plants regenerated from cultured leaf explants (Evans and Sharp, 1983). Therefore, new breeding varieties can be obtained within a short period as a result of somaclonal variation.

Somaclones that have increased resistance to Fiji's disease and Downy mildew have been identified in sugarcane. In potato, somaclones resistant to Alternaria solani toxin and to Phytoothora infestans have been identified. Somaclonal variants have also been identified in tobacco, rice, oats, maize and barley tissue cultures (Larkin and Scowcroft, 1981).

Although somaclonal variation can be advantageously used for selection of genetically different plants, it can also be disadvantageous in the case where one clone is to be propagated. In addition, a few problems or disadvantages still limit the practical use of somaclonal variation. One of these is the fact that somaclones are almost similar stocks that differ by only one gene or a few genes.

If a tissue culture system for passion fruit is established, further work could be done to investigate whether somaclonal variants can be found.

1.4 Transformation

Another possible way of improving on the quality of passion fruit is through transformation. Transformation refers to the transfer and expression of foreign genes in plant cells (Herrera-Estrella and Simpson, 1988). This has become a major tool to carry out gene expression studies and to attempt to obtain improved varieties of potential agricultural or commercial interest (Herrera-Estrella and Simpson, 1988). Plant transformation systems that have been investigated either use Agrobacterium as a vector or use direct gene transfer techniques to transfer foreign genes into plants.

1.4.1 Agrobacterium tumefaciens

Agrobacterium tumefaciens is a Gram-negative soil bacterium that can cause the formation of tumours on certain wounded plants. These bacteria harbour oncogenic plasmids which enable them to induce crown gall tumours (crown gall disease) on most dicotyledonous and some monocotyledonous plants (De Cleene and De Ley, 1976; De Cleene, 1985). All tumour inducing Agrobacteria harbour an extrachromosomal piece of circular DNA called the Ti (Ti=tumour-inducing) plasmid (Zaenen et al., 1974). The size of the Ti plasmid is about 200 kb (Genetello et al., 1977). Agrobacteria devoid of this plasmid are not oncogenic.

A. tumefaciens has a natural capacity to transfer, insert and express a particular segment of its Ti plasmid DNA in the plant cell genome. The segment of the Ti plasmid DNA which is transferred from the bacterium and becomes stably integrated into the plant genome has been called the T-DNA (Transferred DNA) (Chilton et. al., 1977).

A cluster of genes known as vir (virulence) genes, located on the Ti plasmid, outside of the T-DNA, is responsible for the excision and transfer of the T-DNA region of the plasmid from the bacteria to the plant cell (Hooykaas et al., 1984). However, the recognition of susceptible wounded plant cells is mediated by phenolic compounds present in plant exudates (Stachel and Zambrisky, 1986). Lignin is synthesised when wounded plants start healing and lignin precursors are phenolic compounds that are detected by A. tumefaciens and serve as both a chemoattractant for chemotaxis toward the wounded site of the plant and subsequently as an inducer of Ti plasmid inducer vir genes (Kado, 1991). Acetosyringone, sinapinic acid, coniferyl alcohol, caffeic acid, and methylsyringic acid are all inducers of these vir genes (Boulton et al., 1986; Stachel et al., 1986). Vir gene inducers have been reported from wheat cells (Messens et al., 1990) and in maize (Grimsley et al., 1989). Inhibitors of vir gene induction in maize have recently been reported (Sahi et al., 1990).

Plant cells transformed by virulent A. tumefaciens become tumorous. Such cells are able to grow indefinitely, free of inciting bacteria, on media without plant hormones. For normal plant cells to grow and form callus in culture, hormones must be added (Watson et al., 1983) The tumorous character results from the overproduction of the phytohormones, auxin and cytokinin, specified by T-DNA genes. Auxin is formed when two of the T-DNA genes code for enzymes which convert tryptophan into indole-3-acetamide and finally into indole-3-acetic acid. Cytokinin is formed when another gene codes for the enzyme isopentenyl transferase which is responsible for the synthesis of a cytokinin type of plant hormone (Akiyoshi et al., 1984).

Cells of crown gall tumours induced by A. tumefaciens begin to synthesise unusual amino acid derivatives called opines (Morel, 1956). Originally, two groups of opines were

recognised, octopine and nopaline. The octopine family are carboxyethyl derivatives of arginine, which results from the condensation between arginine and pyruvic acid. Arginine is found in all plants. Nopaline and related compounds are dicarboxypropyl derivatives of arginine and ketoglutaric acid (Tempé and Petit, 1982). Further opines have been identified more recently. For example, agropine (Firmin and Fenwick, 1978) which is a bicyclic sugar derivative of glutamic acid, and succinamopine (Chilton et al 1984), a dicarboxypropyl asparagine.

Christou et al (1986), provided evidence that sometimes opines can be found in normal callus and plant tissue as a result of arginine metabolism. The authors detected nopaline in both callus and leaf tissue of soybean and cotton. Presence of opines in callus or plant tissue should therefore only be considered a preliminary indication of transformation by Agrobacterium.

Strains of A .tumefaciens that induce opines can use them as sources of carbon and nitrogen, and the ability, both to induce and to metabolise opines is encoded by Ti plasmids in the bacteria. Host plant cells cannot use these new amino acid derivatives, so the bacterial infection not only causes cells to become tumorous but also subverts the plants' metabolism to making amino acid derivatives that only the bacteria can use as nutrients (Watson et al., 1983).

As such A. tumefaciens is a natural system for gene transfer and vectors have been developed to transfer and express any gene of interest into plants (for a review: see Klee et al 1987). These vectors lack the disadvantages of the virulent A. tumefaciens bacteria i.e. tumour formation and the inability to regenerate normal plants from those tumours.

A. tumefaciens C58 carrying plasmid pTiC58 is a nopaline wild-type strain (Depicker et al., 1980). The T-DNA encodes genes for synthesis of nopaline and agrocinopine (Binns and Thomashow, 1988). Agrocinopine is a phosphorylated sugar derivative. A tumefaciens C58C1 has no Ti plasmid and hence is non-oncogenic. The cured non-tumour inducing strain C58C1

was derived from a single colony of A. tumefaciens C58 by growth at 37°C, which resulted in both the loss of its Ti plasmid and the ability to induce tumours (Van Larebeke et al., 1975).

A. tumefaciens strain C58C1(pTiB6S3) carries plasmid pTiB6S3 and is an octopine-type strain (Petit et al., 1978). This strain was obtained by first curing strain C58 of its Ti plasmid to yield C58C1 and then introducing another Ti plasmid pTiB6S3 from the wild type strain B6S3. C58C1(pTiB6S3) contains two T-DNA's, namely the TL (left) and TR (right) (Thomashow et al., 1980). The TL-DNA encodes one gene known to be involved in opine synthesis, known as the ocs (octopine synthase) gene. This gene codes for octopine synthase, an enzyme that catalyses a reductive condensation between pyruvate and arginine to yield octopine. Three additional genes located in the TR-DNA encode the pathway for the synthesis of two other opines, agropine and mannopine.

A. tumefaciens strains vary in their host range as well as in the time at which tumours appear after infection (Hood et al., 1984). In this regard, A. tumefaciens A281 carrying plasmid pTiBo542 (Hood et al., 1984) is of particular interest because of its ability to induce large early appearing tumours on a wider range of plants than do other strains. Tumours incited by this Ti plasmid produce agropine, leucinopine, mannopine, mannopinic acid, agropinic acid and agrocinopines C and D.

As such, A. tumefaciens A281 has a different chromosomal background as well a different Ti plasmid when compared to the nopaline strain C58 and the octopine strain C58C1(pTiB6S3). The supervirulence of A. tumefaciens A281 is attributed to a 2.5 kb DNA fragment (Jin et al., 1987). This fragment is part of the vir region of the Ti plasmid. The increased virulence of the supervirulent strain A281 was correlated with an increased expression of vir genes (Jin et al., 1987).

1.4.2 Agrobacterium rhizogenes

An alternative tool for gene transfer is Agrobacterium rhizogenes which differs from A. tumefaciens by inducing roots rather than tumours (Chilton et al., 1982; David et al., 1984). Virulent A. rhizogenes strains harbour Ri (Ri=root inducing) plasmids. The Ri

plasmid is as large as the Ti plasmid (200 to larger than 800 kb.) and contains regions homologous to the T-DNA and vir regions (Gelvin, 1990).

Ri plasmids found in A. rhizogenes can be classified into agropine and mannopine-type plasmids (Gelvin, 1990). There appears to exist two mechanisms of hairy root tumourigenesis: one depends upon auxin over production directed by the TR-DNA of certain A. rhizogenes strains, but the other is apparently independent of the transfer and expression of genes directing the biosynthesis of auxin (Gelvin, 1990).

A. rhizogenes like A. tumefaciens can also induce undifferentiated crown gall tumours, either when cytokinin is added during the induction phase (Beiderbeck, 1973) or by infection on some plants (De Cleene and De Ley, 1981).

1.4.3 Transgenic plants

Transgenic plants have been obtained by using A. tumefaciens-based vectors in which the tumour inducing (Ti) genes have been deleted from the T-DNA and replaced with chimeric genes for antibiotic resistance (Horsch et al., 1985). Such selectable markers, for example vectors carrying a chimeric kanamycin resistance, are used to select the transformed callus on kanamycin containing medium.

Transgenic Petunia, tobacco, tomato and many others have been obtained by using various strains of A. tumefaciens (Horsch et al., 1985). Among the monocotyledonous plants, transgenic Asparagus plants have been obtained (Bytebier et al., 1987). Transgenic cucumber plants have been regenerated from the roots induced by a strain of A. rhizogenes (Trulson et al., 1986).

To our knowledge, reports describing a transformation system for P. edulis are lacking. If such a system is described, transgenic passion fruit plants can be obtained in future.

Objectives of the study

1) To establish an *in vitro* tissue culture system for *P. edulis*. If established, the system can be used: (a) to propagate interesting clones of *P. edulis*.

(b) to isolate somaclonal variants,

(c) as a tool in obtaining transgenic plants.

2) To examine if *P. edulis* can be transformed by *A. tumefaciens* and *A. rhizogenes*. If feasible the approach would open the possibility of improving the crop by genetic engineering, for example its resistance to passion fruit woodiness virus.

3) To investigate why passion fruit seeds are dormant. If dormancy could be broken, the period required for seed germination can be reduced.

CHAPTER TWO

Materials and Methods

2.1 Plant material

Three passion fruit cultivars were used: *P. edulis* var. *edulis* (purple variety), *P. edulis* var. *flavicarpa* (yellow variety) and an F1 hybrid (*P. edulis* var. *edulis* X *P. edulis* var. *flavicarpa*; Njue, personal communication). Seeds and plants were obtained from the Kenya Agricultural Research Institute (KARI) research stations at Thika for the purple variety and Mtwapa for the purple and yellow varieties, and the F1 hybrid. The plants were kept outside where they were allowed to grow and serve as a source of different explants, for example stems and leaves.

2.2 Medium

In all the experiments MS (Murashige and Skoog, 1962) medium with doubled vitamin and organic complex was used (Scorza and Janick, 1979). The medium consisted of MS salts plus sucrose, 30 g/l; thiamine.HCl, 0.2 mg/l; pyridoxine.HCl, 1.0 mg/l; nicotinic acid, 1.0 mg/l; glycine, 4.0 mg/l and myo-inositol, 200mg/l. pH was adjusted to 5.8 ± 0.1 using 1.0 N hydrochloric acid and/or 1.0 N sodium hydroxide. 8 g/l agar was added to solidify the medium.

The medium was sterilised by autoclaving at 1 kg/cm^2 and 121°C for 20 minutes and thereafter dispensed into sterile bottles or petri dishes in a laminar air flow cabinet.

2.3 Explant preparations

2.3.1 Seeds

Seeds obtained from ripe fruits were carefully washed in water to remove the pulp and either dried in the sun or at room temperature. Seeds inoculated *in vitro* were surface sterilised in 70% (v/v) ethanol for 2 minutes followed by 30% (v/v) commercial bleach for 20 minutes and then rinsed three times in sterile distilled water and dried using sterile tissue paper. Seeds from immature fruits were obtained after surface sterilisation of whole immature fruits using the procedure described above. The fruits were then cut open and the seeds were removed aseptically.

2.3.2 Leaf explants

Immature leaves excluding buds were excised from the three terminal nodes of the passion fruit plants that had been grown outside. These were then rinsed under running tap water and then surface sterilised in 70% ethanol for 2 minutes followed by 20% commercial bleach for 20 minutes and then rinsed three times in sterile distilled water. Leaf discs (± 1 cm^2) were cut from the sterilised leaves and inoculated onto MS medium supplemented with either auxin or cytokinin or both as described in results.

Nicotiana tabacum (cultivar not known) leaf disc regeneration was performed to check the quality of the MS medium. Leaves were excised from plants grown outside and prepared as described by Tisserat (1985). These were then inoculated onto MS medium supplemented with 0.1 mg/l NAA and 1 mg/l BAP. Shoots that formed were transferred to MS medium without hormones for rooting. The regenerated plants were subcultured every 4 weeks by cutting them into smaller stem segments, each with one leaf. These were then planted in MS medium without hormones.

2.3.3 Stem explants

Stem pieces of passion fruit were excised from the plants that had been grown outside and surface sterilised as described in section 2.3.2. However, the ends of the sterilised pieces (where the cells have been killed) were cut off (Dixon, 1985). The stems were then cut into smaller pieces (± 1 cm long) and inoculated either horizontally or planted vertically into MS medium supplemented with either auxin or cytokinin or both.

2.4 Incubation

All the cultures were incubated under a 16-hours light/8 hours darkness. In case of seeds, a 13-hours light/11 hours dark photoperiod was also used. Light was supplied by 4 fluorescent 40 W lamps. The average temperature of the cultures was 26 ± 2°C.

2.5 Seed germination or breaking of dormancy

Passion fruit seeds were pre-treated as follows:

1) Untreated seeds (control)

2) Seeds soaked in 0.1 M hydrochloric acid for 24 hours then transferred to distilled water for another 24 hours (Khan, 1971)

3) Seeds soaked in water for 48 hours (Chapman, 1963)

4) Seeds soaked in water for 48 hours and then boiled (in water) for 2 minutes (Dell, 1980)

5) Seeds soaked in 500 ppm gibberellic acid (GA3) for 2 days (Khan, 1971). Other seeds (untreated) were incubated on MS medium supplemented with 500 ppm GA3.

6) Seeds soaked in potassium nitrate solution (1 g/l) for 1 hour (Palevitch and Thomas, 1976).

50 seeds from each of the above treatments (except treatment 5) were inoculated on MS medium while 15 seeds from each treatment were sown in soil.

2.6 Transformation procedures

2.6.1 Agrobacterium strains and culture

Five strains of Agrobacterium were used:

1) A. tumefaciens A281

2) A. tumefaciens C58

3) A. tumefaciens C58C1

4) A. tumefaciens C58C1(pTiB6S3)

5) A. rhizogenes ATTC 15834.

All the Agrobacterium strains were obtained through Prof. J.-P. Hernalsteens, Free University, Brussels, Belgium.

A. tumefaciens strains were grown on Minimal A medium (Min A; Miller, 1972) for 3 days at 28°C. A colony from each strain of A. tumefaciens was then grown in 80 ml Luria-Bertani (LB) medium (Miller, 1972) overnight at 28°C on a shaker. A. rhizogenes ATTC

15834 was grown on solid YEB medium (Van Larebeke et al., 1977) at 28°C for 5 days and thereafter grown in liquid YEB medium overnight at 28°C on a shaker. Only freshly grown bacteria were used in all the experiments.

2.6.2 In vitro infection

Leaves from the following plants were surface sterilised as described in section 2.3.2: P. edulis var. edulis, P. edulis var. flavicarpa, F1 hybrid, P. ligularis and P. subpeltata. Tobacco leaves were obtained from in vitro plants. Leaf discs (± 1 cm^2) were cut from the sterilised or sterile leaves (tobacco) and submerged in respective cultures of A. tumefaciens and A. rhizogenes, grown overnight at 28°C. The discs were gently agitated for 1-2 minutes to ensure that all the edges were infected. These were then blotted dry using sterile tissue paper to remove the excess bacteria and incubated on petri plates containing agar-solidified MS medium for 2-3 days to allow the bacteria to grow (Horsch et al., 1985). At least five leaf discs were used for every strain of bacteria.

After 2 days, the leaf discs were transferred to MS medium supplemented with 500 mg/l cefotaxime (Claforan®, Hoechst E.A.), to kill the inciting bacteria. The petri plates were sealed using plastic foil and incubated under a 16-hour light/8-hour dark photoperiod. Subculturing was done every 4-6 weeks when the leaf discs/tumours were transferred to fresh petri plates containing the same medium plus either cefotaxime (500 mg/l) or carbenicillin (500 mg/l).

2.6.3 In vivo infection

Strains of A. tumefaciens A281, A. tumefaciens C58, A. tumefaciens C58C1, A. tumefaciens C58C1(pTiB6S3) and A. rhizogenes ATTC 15834 were grown on solid medium as described in section 2.6.1. Five passion fruit plants that were about 12 weeks old were infected with each strain of A. tumefaciens and A. rhizogenes. All the plants used were of var. edulis.

Fresh wounds were inflicted at each site that was to be infected using a scalpel. For each plant six infections were carried out: one at the cut tip of the stem, three on wounded stem

surface, and two on the leaf. Each plant was infected with one strain of bacteria only. The infection was performed using different sterile toothpicks for each bacterial strain. Bacteria were scooped from the solid plates using a toothpick and applied directly at the wounded site.

Stems were inoculated in four main regions: the cut tip, the youngest part of the stem just below the shoot apex, the middle region of the stem and the oldest region of the stem. Leaves were infected at two sites: at the cut apex of the leaf as well as the mid-vein region at the base of the leaf. The infected plants were observed at least three times a week for any sign of tumour formation.

2.7 Tumour culture

Tumours were excised from the plants after about 4 months and surface sterilised as described in section 2.3.2. Other infected sites that had not developed tumours were also excised and surface sterilised in the same way. All the excised explants were incubated on hormone-free MS medium containing 500 mg/l cefotaxime antibiotic.

Tumours that were large were cut into smaller pieces prior to incubation on hormone-free MS medium. Tumours from different sites were incubated on different petri plates. Small pieces of tumour tissue were cut off from the incubated tissues after two days, and tested for the presence of opines.

2.8 Agropine-mannopine test

Agropine-mannopine test was done according to Leemans et al.(1981). 100-200 mg of tumour tissue was taken and crushed in sterile Eppendorf® tubes. Different sterile splints were used for each tumour tissue. The crushed tumours were then centrifuged in an Eppendorf® centrifuge for 5 minutes. Meanwhile, a Whatman™ 3 MM filter paper measuring 23x18 cm was cut. A baseline (3 cm from the bottom) was marked and a straight line drawn. Seven spots (2 cm apart) were marked on the baseline and spotted with 5 µl of supernatant from each tumour tissue.

The Whatman™ 3MM filter paper was then wetted on both sides in the running buffer, except for the baseline and blotted dry using tissue paper. The running buffer consisted of

formic acid/acetic acid/water (5:15:80 v/v). The paper was then fixed in the electrophoresis chamber and left running, from cathode to anode, for 1 hour at 400 V using the above buffer. After 1 hour the paper was removed from the electrophoresis chamber and immediately blotted dry using tissue paper before hanging it in a hood overnight for further drying.

Once dry, the following staining procedure was adopted (Trevelyan et al., 1950): the paper was dipped in 0.4% silver nitrate dissolved in acetone for 30 seconds with gentle shaking. The paper was then placed back on the clothes' line for a few seconds while the following solution was being prepared: 10 ml 20% potassium hydroxide plus 90 ml ethanol (technical). The paper was then dipped with the written part down with one fluent movement in this solution and left to react for 30 seconds to 1 minute. The paper which was now stained, was hung again on the clothes' line for 10 minutes.

The stain was then fixed with an acid-fixing solution for 30 seconds. This solution consisted of sodium thiosulphate (150 g/l) and sodium bisulphite (15 g/l). The fixed stain was rinsed in running water for several hours and was then finally dried.

CHAPTER THREE

Results

3.1 Seed germination

Seeds of passion fruit that had received other pre-treatments than the standard pre-treatment of soaking in water for 48 hours, showed no improvement as far as germination in soil was concerned (see table 4). The first batch of seeds to germinate were those that had been pre-treated by soaking in water for 48 hours. These seeds started to germinate after 21 days. The next batch of seeds to germinate were those that had been pre-treated by soaking in 0.1 M. hydrochloric acid followed by transfer to distilled water for another 24 hours. These seeds started to germinate after 28 days.

The rest of the seeds namely those that had been pre-treated by soaking in 2 g/l potassium nitrate solution for 48 hours and those that had been pre-treated by soaking in 500 ppm gibberellic acid (GA3) for 48 hours germinated in 4-5 weeks. None of the seeds that had been soaked in water for 48 hours and then boiled in water for 2 minutes germinated (see table 4).

None of the seeds that had received the same pre-treatments as above and were inoculated *in vitro* germinated, even after being incubated under 2 different kinds of photoperiod; namely 16 hours light/8 hours dark and 13 hours light/11 hours dark. However, seeds that had been scarified or whose seed coats had been broken and were inoculated *in vitro* germinated (table 5). Such seeds, however, showed poor development as most of them were unable to grow normally. Scarified seeds inoculated *in vitro* after pre-treatment with GA3 also germinated. Scarified seeds inoculated on MS medium supplemented with 500 ppm GA3 started to germinate after 14 days. Eleven out of 25 seeds (44%)had germinated by day 48. Scarified seeds soaked in 500 ppm for 1 hour then inoculated on MS medium started to germinate after 25 days. Only 10 out of 25 seeds (40%) germinated within 48 days. In both cases, the seedlings were abnormal, showing unusually elongated stems.

Table 4: In vivo seed germination.

Variety	Treatment	N sown	N germ.	%	Remarks
edulis	untreated	15	13	86.7	germination started after 42 days when 2 seeds germinated
edulis	seeds soaked in water for 48 hours	15	15	100	first seed germinated after 21 days
edulis	seeds soaked in 0.1 M. HCl for 24 hrs. plus distilled water for 24 hrs.	15	11	73.3	first seed germinated after 28 days
edulis	seeds soaked in 500 ppm. GA3 for 2 days	15	12	80.0	first two seeds germinated after 34 days
edulis	seeds soaked in KNO3 solution (2 g/l) for two days	15	14	93.3	first seed germinated after 36 days
edulis	seeds soaked in water for 48 hrs. and boiled for 2 minutes	15	0	0	no germination
flavicarpa	seeds soaked in water for 48 hrs.	15	11	73.3	first two seeds germinated after 42 days
F1 hybrid	seeds soaked in water for 48 hrs.	15	13	86.7	first seed germinated after 42 days

Legend: N sown: number of seeds sown

N germ.: number of seeds germinated in 8 weeks

%: germination percentage

Table 5: In vitro seed germination (var. edulis)

Treatment	No.seeds	No.germ.	Remarks
seeds soaked in water for 48 hrs.	10	-	no germination observed
scarified seeds soaked in water for 48 hrs	10	1	germination observed after 5 weeks
scarified seeds inoculated on MS medium supplemented with 500 ppm GA3	25	11	first seed germinated after 14 days
scarified seeds soaked in 500 ppm GA3 and then inoculated on MS medium	25	10	first seed germinated after 25 days
seeds whose seed coat was removed	5	4	germination observed after 7 days
seeds whose seed coat was removed from one side only	5	3	germination observed after 7 days
seeds soaked in water for 48 hrs and inoculated on 1/2 MS	16	-	no germination

Legend: No. seeds: total number of seeds inoculated

No. germ: number of seeds germinated in 5 weeks

Seedlings of var. edulis grew into normal plants which matured in 7-8 months when they began to flower and set fruit. Flowering occured throughout the year as the plants were watered regularly, although on a small scale. However, the main flowering and fruiting season occured after about 10 months from the time of transplanting. Seedlings of var. flavicarpa and the F1 hybrids also grew into normal plants. However, these plants were unable to set any fruits or flowers.

3.2 Initiation of callus from immature seeds

Six different media were tested for callus initiation from immature seeds. Auxins used included NAA whose concentration ranged from 0.5-4 mg/l, and 3 mg/l IAA. The cytokinin supplement was BAP whose concentration ranged from 0.5-1.5 mg/l. All the immature seeds were from var. edulis. The results are summarised in table 6.

In one experiment using MS media supplemented with 1 mg/l NAA and 0.5 mg/l BAP, two replicates each with 25 immature seeds were used. A large proportion of these seeds became swollen and the seed coat was ruptured within 3 days. Seventeen seeds (35%) changed colour from white to green within 5 days. Twenty-eight seeds (56%) formed callus within 18 days. Callus began to appear on day 5 and grew rapidly for about 7 days after which further growth became arrested. If subculturing to the same medium was done after 2 instead of 4 weeks, fast growth of the callus was evident during the first week of subculture only.

Most of the calluses observed were either arising from the seed coat or from the seed stalk region. There was no evidence of callusing from the seed embryo. Callus could be maintained alive on MS medium containing 1 or 2 mg/l NAA and 0.5 mg/l BAP for more than 3 months provided subculturing was done every 4 weeks.

Two media were tested for regeneration: one of the media was supplemented with 0.5 mg/l NAA and 1 mg/l BAP while the other medium contained 0.1 mg/l NAA and 1 mg/l BAP. No regeneration was observed in either of the two cases. No regeneration was also observed in any of the subsequent subcultures when the calluses were subcultured onto the same medium as was used for callus initiation.

Table 6: Initiation of callus from immature seeds.

NAA (mg/l)	IAA (mg/l)	BAP (mg/l)	No. of replic.	No. of expl.	Remarks
0.5	-	1.5	1	25	5 seeds (20 %) callused within 18 days
1	-	0.5	2	50	28 seeds (56%) callused within 18 days
2	-	0.5	2	50	32 seeds (64%) callused in 18 days
3	-	0.5	1	25	11 seeds (44%) callused in 18 days
-	3	0.5	2	90	47 seeds (52%) callused in 18 days
4	-	0.5	1	25	12 seeds (48%) callused in 18 days

Legend: No. of replic.: number of replicates used in the experiment

No. of expl.: total number of explants used

3.3 Initiation of callus and shoots from stem explants of var. edulis

Six different media were tested for initiation of callus from stem explants of var. edulis. Two different media were tested for shoot induction. The auxin used was NAA whose concentration ranged from 0.1-4 mg/l while the cytokinins used included: 0.5-2 mg/l BAP and 2 mg/l KIN. The results are summarised in table 7.

In one experiment 25 stem pieces were used to initiate callus from stem explants of var. edulis using MS medium supplemented with 2 mg/l NAA and 0.5 mg/l BAP. In this experiment, 13 stem segments were planted vertically in the medium while 12 were inoculated horizontally. Sixteen out of 25 explants (64%) formed callus within 18 days. The response was better in stem explants that had been inoculated horizontally on the medium. Callus began to grow from the cut edges of the stem within 5 days (see fig. 1).

In all the stems in which callus growth was taking place, the growth was starting from the upper side of the cut edge of the stem. This would then continue to grow until the whole of the cut surface forms callus. The calluses were green in colour and could not be easily separated from the stem explant.

These calluses failed to regenerate shoots or roots when subcultured onto MS medium supplemented with 0.5 mg/l NAA and 1 mg/l BAP. No regeneration was obtained when MS medium supplemented with 0.5 mg/l NAA and 2 mg/l BAP was used. Root regeneration was observed in a few of the stems when the calluses were subcultured onto the same medium as used for callus initiation, namely MS medium supplemented with 2 mg/l NAA and 0.5 mg/l BAP. In one experiment, 4 of the 15 calluses (26.7%) formed roots during week 7.

In another experiment, 25 stem pieces of var. edulis were inoculated on MS medium supplemented with 0.2 mg/l NAA and 2 mg/l BAP (Muralidhar and Mehta, 1982). Twelve of these pieces were stem segments with nodes while the rest lacked nodes. Eleven out of the 25 stem pieces (44%) formed callus within 18 days. Seven out of the 12 stem pieces (58.3%) with nodes developed shoots from the node after 12 days. Stem pieces without nodes failed to develop shoots. Stem pieces with nodes, incubated on medium without hormones, also failed to

Table 7: Initiation of callus and shoots from stem explants of var. edulis

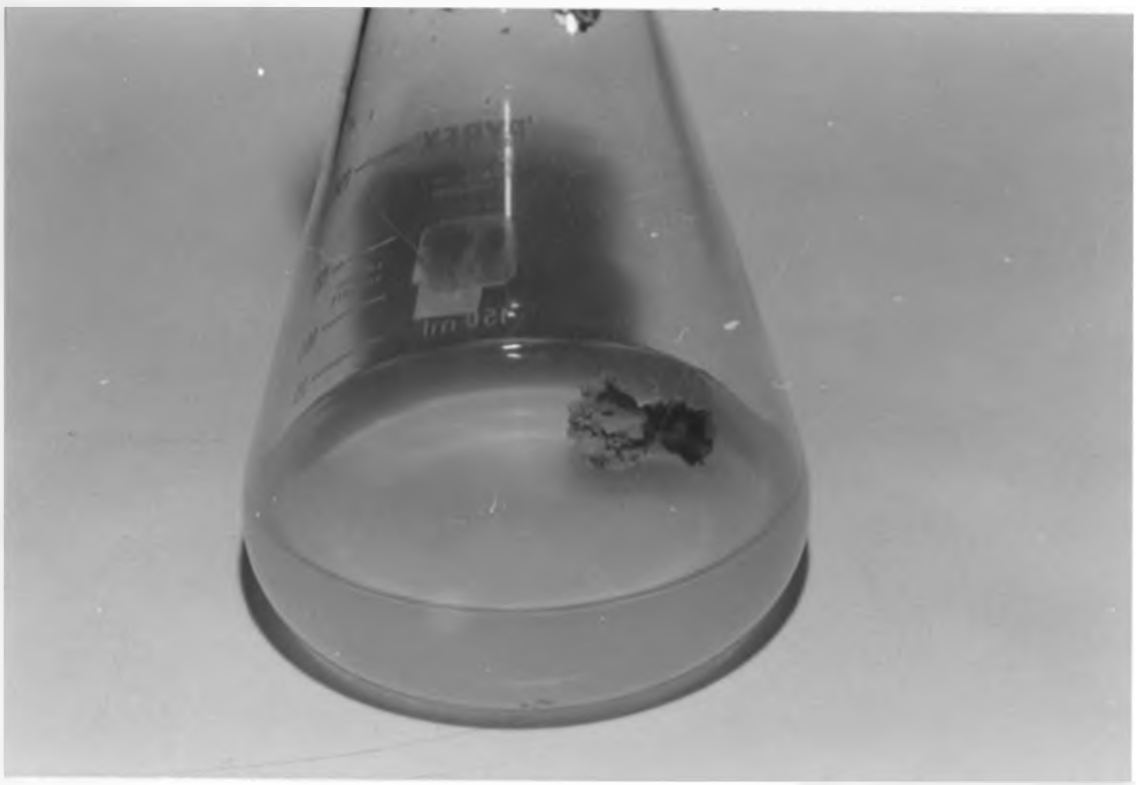
NAA mg/l	BAP mg/l	KIN mg/l	No. of replic	No. of expl.	No. lost	Remarks
0.1	1	-	1	9	2	no callus, buds appear from nodal regions of the stems in 5 days
0.5	1.5	-	1	8	-	no callus, no shoots
2	0.5	-	2	65	15	28 stems (56%) callused in 18 days
3	0.5	-	1	40	-	21 stems (52.5%) callused in 18 days, no shoots
4	0.5	-	1	40	-	16 stems (40%) callused in 18 days, no shoots
-	-	2	1	15	-	2 stems (40%) callused after 14 days, no shoots
-	2	-	1	25	-	5 stems formed shoots while 11 (44%) callused in 18 days

Legend: No. of replic.: total number of replicates used in the experiment

No. of expl.: total number of explants used

No. lost: total number of explants lost due to infection or death

Figure 1: 6-week old callus initiated from a stem segment of var. edulis on MS medium supplemented with 2 mg/l NAA and 0.5 mg/l BAP



develop shoots. Of the 7 stems that developed shoots, 5 were lost due to fungal infection. The rest were transferred to MS medium without hormones for 2 weeks as recommended by Kantharajah and Dodd (1990). Rapid growth resumed when the 2 shoots were transferred to MS medium without hormones. Roots developed on one of the shoots within the next 4 weeks. However, the roots were arising directly from the stem explant and not from the newly formed shoot.

Similar results were obtained when stem pieces of var. edulis were inoculated on MS medium supplemented with 2 mg/l BAP (Kantharajah and Dodd,1990). Out of 25 explants,13 had nodes while the rest lacked nodes. Five out of 13 explants with nodes (38.5%) formed shoots within 4 weeks (see fig. 2), while 11 of the total number of explants (44%) formed callus within 18 days. No shoots were observed on the explants without nodes. Most of the shoots were lost after being subcultured due to fungal infection.

3.4 Initiation of callus and shoots from stem explants of var. flavicarpa

Five different media were tested for callus initiation from stem explants of var. flavicarpa. Ten different media were used to try and regenerate callus that was obtained from the stems. Five calluses were used in every experiment. One medium was tested for direct shoot induction from the stems. The results of callus initiation and shoot induction are summarised in table 8.

In one of the experiments using MS medium supplemented with 2 mg/l NAA, 25 stem pieces were used. Three of these (12%) were lost due to fungal infection while 2 (8%) were lost due to death within the first 14 days, leaving a total of 20 explants. Seventeen of the remaining explants (85%) formed callus within 14 days. Callus began to appear after 5 days and was subcultured after 4 weeks. Figure 3 shows callus 17 days after initiation and figure 4 illustrates 7 weeks old callus.

Figure 2: 6-week old shoot from a nodal segment of var. edulis on MS medium supplemented with 2 mg/l BAP

Table 8: Initiation of callus and shoots from stem explants of var. flarvicarpa

NAA (mg/l)	2,4-D (mg/l)	BAP (mg/l)	No. of replic.	No. of expl.	No. lost	Remarks
2	-	-	3	125	25	callus with globular structures observed after 4 days, no roots
3	-	-	2	92	8	callus with globular structures observed after 4 days, no roots
4	-	-	1	42	8	callus with globular stuctures observed after 4 days, no roots
-	2	-	1	15	-	compact callus observed within 4 days
-	-	2	1	25	7	little compact callus observed within 14 days, shoots observed within 4 weeks

Legend: No. of replic: total number of replicates used in the experiment

No. of expl. : total number of explants used in the experiment

No. lost: total number of explants lost due to infection or death

Figure 3: 17-day old calluses initiated from stem segments of var. flavicarpa on MS medium supplemented with 2 mg/l NAA

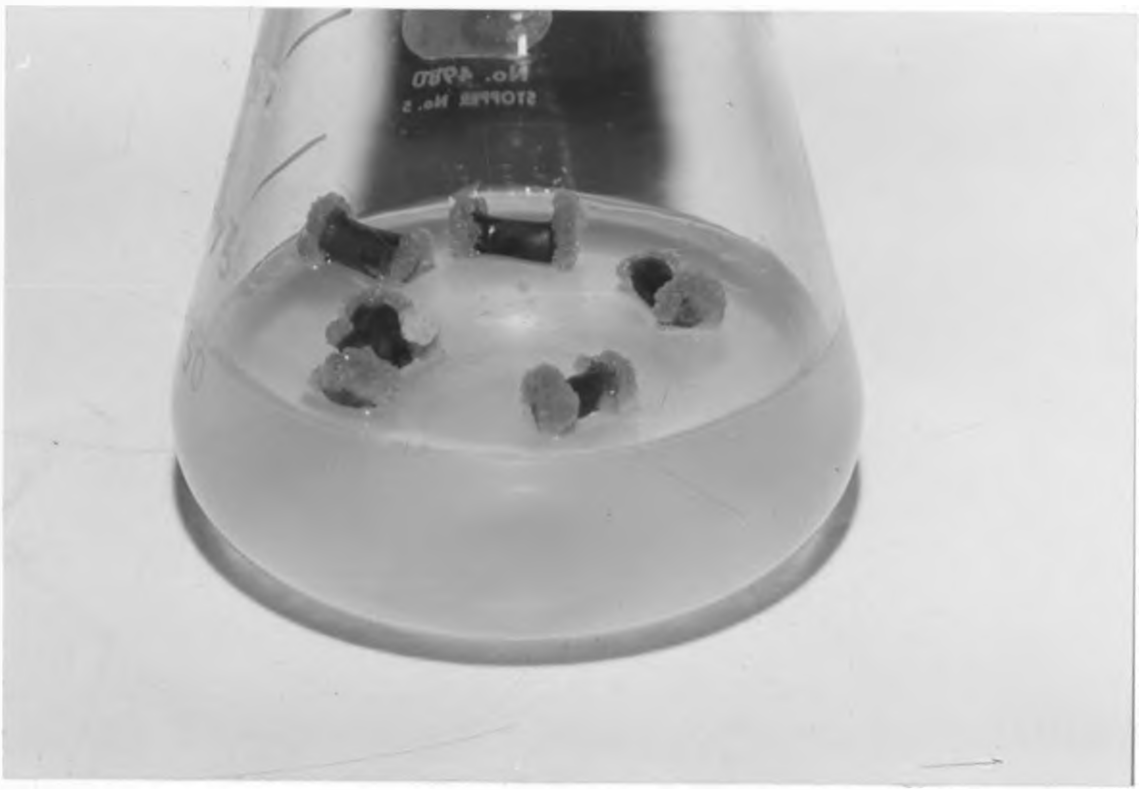
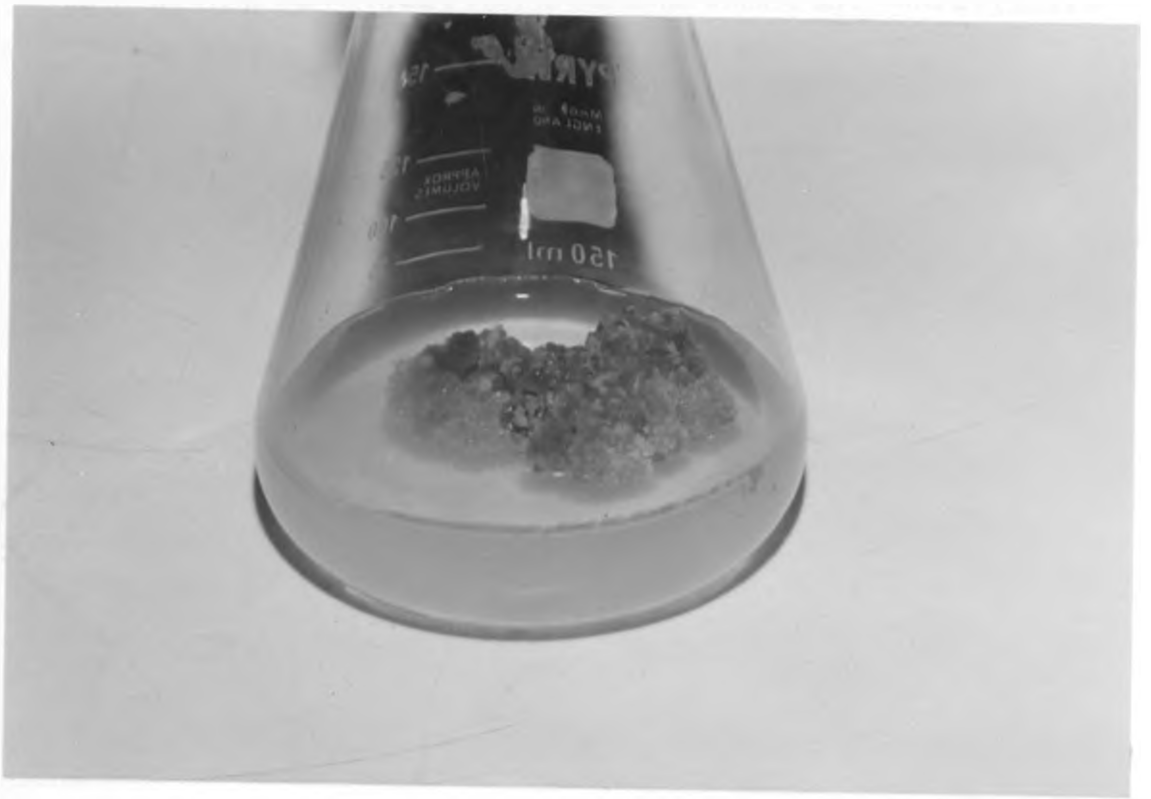


Figure 4: 7-week old callus derived from stem segments of var. flavicarpa growing on MS medium supplemented with 2 mg/l NAA



In another experiment for callus initiation from stem explants of var. flavicarpa, comparison was made among MS media supplemented with 2 mg/l NAA and 3 mg/l NAA in which 50 explants were used per treatment (table 8). For those inoculated on MS medium supplemented with 2 mg/l NAA, 22 (44%) formed callus within 6 days, while 20 (40%) of the explants inoculated on medium with 3 mg/l NAA formed callus within the same period. Further comparison was made among MS media supplemeted with the following concentrations of hormones: 2 mg/l NAA, 3 mg/l NAA and 4 mg/l NAA. A total of 42 explants were used in each experiment. Seventeen of the 42 pieces of stems (40.48%) inoculated on medium with 2 mg/l NAA had formed callus by day 6. For those on medium with 3 mg/l NAA, 18 (42.86%) had formed callus by day 6. Callus formation was observed on 16 of the 42 stem pieces (38.09%) inoculated on medium with 4 mg/l NAA by day 6. The calluses that formed on MS medium supplemented with NAA appeared to have organised structures which looked like embryos.

In a different experiment for callus initiation, 15 stem explants of var. flavicarpa were inoculated on MS medium supplemented with 2 mg/l 2,4-D. All the 15 explants (100%) formed callus within 7 days. The calluses were white in colour. However, browning of the calluses was observed as from day 9 and they all died within 14 days.

In another experiment, 25 stem pieces of var. flavicarpa were inoculated on MS medium supplemented with 2 mg/l BAP(Kantharajah and Dodd,1990). Seven (28%) were lost due to fungal and bacterial infection. Eleven of the 18 remaining stems (61.1%) formed callus within 14 days. No shoots were observed during this period. Callus formed in this case was white in colour and compact. Five stem pieces (27.8%) developed shoots within 4 weeks. However, such shoots were only observed on stem pieces that had nodes. Those without nodes failed to develop shoots within the first 4 weeks although they developed callus. Such stem pieces however, developed small buds when subcultured onto the same medium (containing 2 mg/l BAP), within 4 weeks. These buds developed at one cut end that was not callusing, although they failed to grow after separation from the stem and subsequent subculture onto MS

medium with 2 mg/l BAP. Callus from the stems also failed to regenerate shoots after separation from the stems and subsequent subculture onto medium containing 2 mg/l BAP.

Attempts to regenerate callus with globular structures derived from stem explants of var. flavicarpa were unsuccessful. There was no shoot regeneration when callus was subcultured onto MS medium supplemented with 0.1 mg/l NAA and 1 mg/l BAP. However, the 5 calluses with globular structures subcultured onto this medium continued to grow and were green in colour. Calluses subcultured onto MS medium without hormones also failed to regenerate and 1 out the 5 calluses died within 18 days while the rest died within 28 days. There was no evidence of differentiation when MS medium supplemented with 0.2 mg/l NAA and 0.5 mg/l BAP (Muralidhar and Mehta,1982).was used. Five calluses were subcultured onto this medium. The globular structures which were clearly evident when the calluses were subcultured became less organised within the next 4 weeks.

No further differentiation was observed when callus with globular structures was subcultured onto MS medium supplemented with a low concentration of either auxin or cytokinin. For example, when medium supplemented with 0.1 mg/l NAA was used, the rate of growth of the 5 calluses was slow when compared to the calluses that were growing on MS medium supplemented with 2 mg/l NAA. No differentiation was also observed in any of the 5 calluses with globular structures subcultured onto medium supplemented with 0.1 mg/l NAA and 2 mg/l casein hydrolysate. Response was poor on this medium and only 2 out of 5 calluses were still alive after 18 days. Similarly, no differentiation was obtained when callus with globular structures was subcultured onto MS medium supplemented with 0.1 mg/l BAP. One callus died within 18 days while the other 4 died after 4 weeks. Callus subcultured onto MS medium supplemented with 0.1 mg/l BAP and 2 mg/l casein hydrolysate also failed to regenerate. The rate of growth was slow in all the 5 calluses subcultured onto this medium and they all died after 4 weeks.

No shoot regeneration was observed when callus with globular structures was subcultured onto MS medium supplemented with 0.5 mg/l zeatin. However, all the 5 calluses continued to grow for 3 weeks after which they turned brown and died. Calluses subcultured onto MS medium supplemented with 1 mg/l KIN also failed to regenerate. The 5 calluses continued to grow but eventually turned brown and died after 16 days. There was no root regeneration when callus was subcultured onto MS medium supplemented with 0.5 mg/l IBA (Muralidhar and Mehta,1986).

Callus with globular structures from stem explants of var. flavicarpa continued to grow when subcultured onto MS medium supplemented with 2 mg/l NAA, provided subculturing was done every 4 weeks. This medium was used to maintain a stock of actively growing calluses. However, the potential for active growth started to diminish after the third subculture i.e. after about 12 weeks. No root regeneration was observed in any of the subsequent subcultures. If subculturing was not done after 4 weeks, the initiating explant would turn brown and die during the sixth week.

3.5 Initiation of callus, roots and shoots from leaf disc explants of var. edulis

Nine different media were tested for initiation of callus, roots and shoots from leaf disc explants of var. edulis. The results are summarised in table 9.

No callus was obtained from leaf discs of var. edulis when any of the 9 different media were used (see table 9). The only response noted was the curling of the leaves which was more prominent on those on MS medium supplemented with 2 mg/l NAA and 0.5 mg/l BAP. Most of the leaf discs used would remain alive for 5-6 weeks after which they would turn brown and die. Subculturing the leaf discs onto the same medium after 2 or 4 weeks did not result in any callus, root or shoot formation.

Table 9: Initiation of callus, roots or shoots from leaf disc explants of var. Edulis

NAA (mg/l)	BAP (mg/l)	2,4-D (mg/l)	Kinetin (mg/l)	No. of replic.	No. of expl.	Remarks
0.1	1	-	-	2	65	no callus, no roots, no shoots
0.5	1	-	-	1	30	no callus, no roots, no shoots
1	0.5	-	-	4	80	no callus, no roots, no shoots
1	1	-	-	5	105	no callus, no roots, no shoots
2	0.5	-	-	1	10	no callus, no roots, no shoots
3	0.5	-	-	3	55	no callus, no roots, no shoots
-	2	-	-	1	50	no callus, no roots, no shoots
-	2	-	0.5	1	50	no callus, no roots, no shoots
-	2	2	-	2	50	no callus, no roots, no shoots

Legend: No. of replic. : total number of replicates used

No. of expl.: total number of explants used

3.6 Initiation of callus, roots or shoots from leaf discs of var. flavicarpa

Seven different media were tested for initiation of callus, roots or shoots from leaf disc explants of var. flavicarpa. The results are summarised in table 10.

In the initial experiment in which 10 leaf discs of var. flavicarpa were inoculated on MS medium supplemented with 2 mg/l NAA, 3 of the 10 leaf discs formed callus within 4 weeks. No roots were observed. In a repeated experiment 47 leaf discs were used. Callus was observed on day 4 when 4 out of 47 leaf discs formed callus. The number of calluses increased to 16 out of 46 by day 7. A total of 5 leaf discs were lost, 3 due to fungal infection and 2 due to death. Death occured after 26 days and the affected leaf discs had not formed callus. Roots began to appear on day 18. Only leaf discs that had formed callus formed roots (see fig. 5). By the end of the fourth week, 23 out of 42 leaf discs (54.7%) had formed callus while 5 (11.9%) of the cultures had formed roots.

In another experiment in which 50 leaf discs were inoculated on MS medium supplemented with 2 mg/l NAA and 0.5 mg/l kinetin, (Desai and Mehta,1985). Twenty two of these (44%) had already formed callus by day 7. Callus began to appear on day 4. Fifteen leaf discs (30%) were lost due to bacterial and fungal infection. All the remaining 35 leaf discs (100%) had formed callus by day 26. Roots began to appear on day 13 and by day 26, 23(65.7%) of the cultures had formed roots.

When leaf discs of var. flavicarpa were inoculated on MS medium supplemented with 2 mg/l NAA, 2 mg/l IAA and 0.4 mg/l KIN (Desai and Mehta,1985), only callus was observed. Callus formation was observed on day 4 when 14 out of 48 (29.2%) of the cultures had formed callus. By day 22, 35 out of 46 leaf discs (76.1%) had formed callus. Four leaf discs were lost, 2 due to fungal infection and 1 each due bacterial infection and death. Leaf discs that had not formed callus turned brown within 28 days and died later on.

Table 10: Initiation of callus, roots or shoots from leaf disc explants of var. flavicarpa

NAA (mg/l)	BAP (mg/l)	KIN (mg/l)	2,4-D (mg/l)	IAA (mg/l)	No. of replic.	No. of expl.	No. lost	Remarks
2	-	-	-	-	2	57	5	callus and roots
2	-	0.5	-	-	1	50	15	callus and roots
2	-	0.4	-	2	1	50	4	callus only
-	-	-	2	-	1	20	-	little callus on mid vein
-	2	-	2	-	2	25	-	no callus, no shoots
-	2	-	-	-	3	150	18	little callus and shoots
-	2	0.5	-	-	3	150	38	little callus and shoots

Legend: No. of replic.: total number of replicates used

No. of expl.: total number of explants used

No. lost: total number lost due to infection or death

Figure 5: 47-day old leaf disc of var. flavicarpa showing callus and roots on MS medium supplemented with 2 mg/l NAA

When leaf discs of var. flavicarpa were inoculated on MS medium supplemented with 2 mg/l 2,4-D, callus was observed on mid-vein only. There were no shoots or roots. Leaf discs inoculated on MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BAP (Muralidhar and Mehta, 1986) failed to form callus or shoots. The leaf discs only enlarged in size.

Leaf discs of var. flavicarpa inoculated on MS medium supplemented with 2 mg/l BAP, formed callus and shoots. In one of the experiments, a total of 50 leaf discs were used (table 10). Thirty-three of them (66%) formed callus within 21 days. However, the callus was much smaller in size when compared with calluses that were induced on medium containing 2 mg/l NAA. Seven out of 46 (15.2%) of the leaf discs formed shoots within 25 days. Shoots began to appear after 18 days and were 10-15 mm long after 38 days. Figure 6 shows 5-day old leaf discs of var. flavicarpa on MS medium supplemented with 2 mg/l BAP for shoot induction. Figure 7 shows 40-day old shoots regenerating from leaf discs of var. flavicarpa on MS medium supplemented with 2 mg/l BAP. Most leaf discs had 1-2 shoots. Four leaf discs were lost, 3 due to death and 1 due to fungal infection. Leaf discs that had not formed shoots but were still healthy by day 28 were subcultured onto the same medium (containing 2 mg/l BAP). A total of 15 leaf discs were subcultured and 7 of these (46.7%) formed shoots within 2 weeks after transfer. Shoots started to appear on the 9 th day.

In a repetition of the same experiment as above, 50 leaf discs of var. flavicarpa were used. Five of these (10%) were lost due to bacterial and fungal infection within the first 8 days. Of the remaining 45 leaf discs, 12 (26.7 %) formed shoots within 28 days. Buds started to appear on some of the leaf discs on day 13 but definite shoots were observed after 20 days. Most of the leaf discs had 1-2 shoots.

Similar results were obtained from leaf discs of var. flavicarpa inoculated on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l kinetin (Desai and Mehta, 1985).. In one of the experiments, a total of 50 leaf discs were used. Four of them (8%) were lost due to fungal infection. Of the remaining 46 leaf discs, 24 (52.2%) had formed callus within 21

Figure 6: 5-day old leaf discs of var.flavicarpa on MS medium supplemented with 2 mg/l BAP for shoot induction

Figure 7: 40-day old shoots regenerating from from leaf discs of var flavicarpa on MS medium supplemented with 2 mg/l BAP.

days. By the end of 28 days, 15 out of 44 leaf discs (32.6%) had formed shoots although a few of the shoots failed to develop stems.

In another experiment using MS medium supplemented with 2 mg/l BAP and 0.5 mg/l kinetin, 13 out of 21 leaf discs (61.9%) formed shoots within 28 days. Shoots began to appear after 18 days. Most of the leaf discs had 2-3 shoots. In both experiments, that is using media supplemented with 2 mg/l BAP or 2 mg/l BAP and 0.5 mg/l kinetin, the shoots arose directly from the explant. There was no evidence of shoots arising from callus. Most of the shoots developed from the cut edges of the leaf discs or from regions near them. Further growth of the shoots was realised by subculturing them onto MS medium with 0.1 mg/l BAP, for 2-4 weeks. Most leaf discs differentiated into cultured plantlets with stem and leaves.

3.7 Plantlets

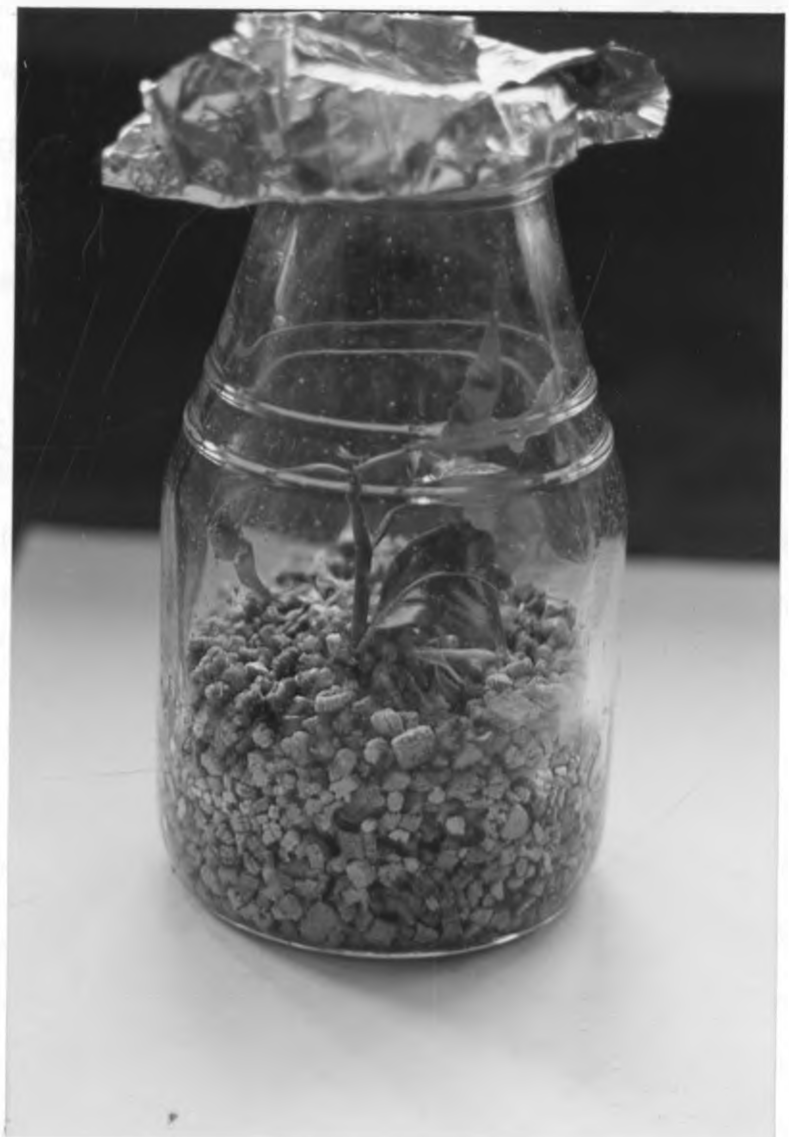
Plantlets with distinct root and shoot systems, were obtained from leaf discs of var. flavicarpa after the shoots were induced to root on MS medium supplemented with 0.1, 0.5 and 1 mg/l NAA (see fig. 8). Roots were observed on 2 of the 4 shoots subcultured onto MS medium supplemented with 0.5 mg/l NAA after 13 days. One of the 4 shoots subcultured onto MS medium with 0.1 mg/l NAA was induced to root within the same period. Rooting occured readily on the shoots that were about 10 mm long as opposed to those that were about 30 mm long. One of the 2 shoots that were about 30 mm long and were subcultured onto MS medium supplemented with 0.5 mg/l NAA started to form roots after 23 days. However, shoots of about the same size (30 mm long), that were subcultured onto MS medium supplemented with 1 mg/l NAA (Kantharajah and Dodd,1990) formed roots after about 17 days. No roots were observed on any of the shoots subcultured onto MS medium supplemented with either 1 mg/l IAA or 0.5 mg/l IBA.

The plantlets were transferred to sterile vermiculite after 8-10 weeks and watered regularly with half-strength MS medium. Figure 9 shows an 8-week old plantlet ready for transfer to vermiculite, while figure 10 shows the same plantlet after transfer to vermiculite. This stage was to prepare the plantlets for soil conditions.

Figure 8: 8-week old plantlet derived from a leaf disc of var. flavicarpa on MS medium supplemented with 0.1 mg/l NAA for root induction

Figure 9: 8-week old plantlet derived from a leaf disc of var. flavicarpa ready for transfer to vermiculite

Figure 10: 8-week old plantlet derived from a leaf disc of var. flavicarpa after transfer to vermiculite



In a control experiment, shoots developed on leaf discs of Nicotiana tabacum inoculated on MS medium supplemented with 0.1 mg/l NAA and 1 mg/l BAP within 24 days. All the 25 leaf discs used in this experiment developed shoots (see fig. 11). The shoots developed into plantlets after being subdivided and inoculated on MS medium without hormones. Roots developed within 5 days of transfer to MS medium.

3.8 In vitro growth of shoots

The tobacco plantlets obtained above were frequently subcultured by cutting the stems into smaller fragments each with one leaf. These were then planted into MS medium without hormones, where they grew into normal plants with roots. They served as a source of sterile material (fig. 12).

Attempts to grow passion fruit plants in vitro starting from stem explants were unsuccessful. When stem sections including axillary buds were cut, surface sterilised and planted into MS medium, no shoot growth was observed in any of the five stems used.

3.9 Infection with wild type Agrobacterium strains

3.9.1 In vitro infection of leaf discs

Tumours were observed on 6 leaf discs out of 10 of var. flavicarpa infected with A. tumefaciens A281 within 6 days. Some of the tumours were growing on the leaf edges while others were growing on the surface of the leaf discs. Tumours were also observed on the leaf discs of the F1 hybrid infected with the same strain of bacteria within 5 days. The tumours formed in both cases were growing rather slowly. Some of the leaf discs, especially those that had not formed tumours, started to turn brown after 20 days. Leaf discs of var. edulis infected with A. tumefaciens A281 started to form tumours after about 38 days. Only 2 leaf discs out of 5 formed tumours (see table 11).

Figure 11: 4-week old leaf disc of N. tabacum showing shoot regeneration

Figure 12: 7-week old plantlet of N. tabacum growing on MS medium without hormones

Table 11: *In vitro* infection of leaf discs

Agrobacterium strain	Variety	No. of replic.	No. of expl.	Remarks
A. tumefaciens A281	*flavicarpa*	2	10	tumours observed on 6 leaf discs within six days
	F1	2	10	tumours observed on 4 leaf discs within 5 days
	edulis	2	10	tumours observed on 2 leaf discs after 38 days
	tobacco	2	10	tumours observed on all the 10 leaf discs within 9 days
A. tumefaciens C58C1pTiB6S3	*flavicarpa*	2	10	no tumours
	F1	2	10	no tumours
	edulis	2	10	no tumours
	tobacco	2	11	tumours
A. tumefaciens C58	*flavicarpa*	2	10	no tumours
	F1	2	10	no tumours
	edulis	2	10	no tumours
	tobacco	2	10	no tumours
A. tumefaciens C58C1	*flavicarpa*	2	10	no tumours
	F1	2	10	no tumours
	edulis	2	10	no tumours
	tobacco	2	10	no tumours
A. rhizogenes ATCC15834	*flavicarpa*	1	5	tumours, one root
	F1	1	5	tumours, no roots
	edulis	1	5	no tumours, no roots
	tobacco	1	5	roots

Legend: No. of replic.: total number of replicates used

No. of expl.: total number of explants used

Tobacco leaf discs were also infected with A. tumefaciens A281 to serve as a control experiment. Tumours were observed on the leaf discs of Nicotiana tabacum infected with A. tumefaciens A281 (fig. 13). Most of the 10 leaf discs used formed tumours within 10 days. The tumours were growing fast. All the leaf discs of P. ligularis and P. subpeltata infected with A. tumefaciens A281 died during the first week of incubation. Death was as a result of the sterilisation procedure used.

No tumours were observed on the leaf discs of var. edulis, var. flavicarpa and the F1 hybrid infected with A. tumefaciens C58C1(pTiB6S3). However, tobacco leaf discs infected with the same strain of A. tumefaciens started to form tumours after 21 days. Two out of the 5 leaf discs that had formed tumours developed shoots. Leaf discs of P. ligularis and P. subpeltata infected with A. tumefaciens C58C1(pTiB6S3) died during the first week of incubation.

No tumours were observed on the leaf discs of var. edulis, var. flavicarpa and the F1 hybrid infected with A. tumefaciens C58. No tumours were observed on the leaf discs of Nicotiana tabacum infected with A. tumefaciens C58. However, in an earlier experiment using leaf discs of N. tabacum, shoots were observed on 2 of the 5 leaf discs infected with A. tumefaciens C58.

A control experiment was done using A. tumefaciens C58C1 which lacks a Ti plasmid and is thus non-tumourigenic. No tumours were observed on the leaf discs of var. edulis, var. flavicarpa, F1 hybrid and N. tabacum infected with A. tumefaciens C58C1.

Leaf discs of var. flavicarpa infected with A. rhizogenes ATCC 15834 started to form tumours after 6 days (fig. 14). Nine leaf discs formed tumours. Only one root was observed arising from one of the tumours after 9 weeks. However, the leaf discs turned brown and died after this period. Tumours were observed on leaf discs of the F1 hybrid infected with A. rhizogenes ATCC 15834 after 10 days. No roots were observed. No tumours or roots were observed on leaf discs of var. edulis infected with the same strain of A rhizogenes (table 11).

Figure 13: 23-day old leaf discs of N. tabacum infected with A. tumefaciens A281 showing formation of tumours

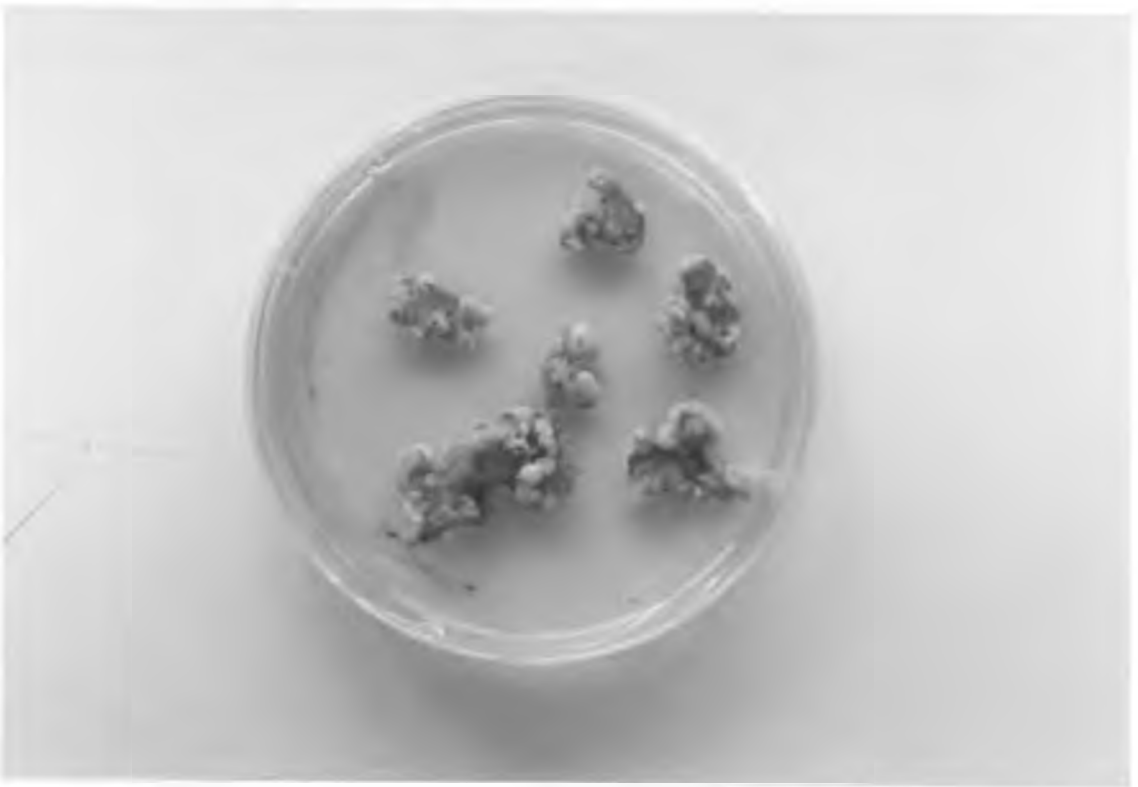
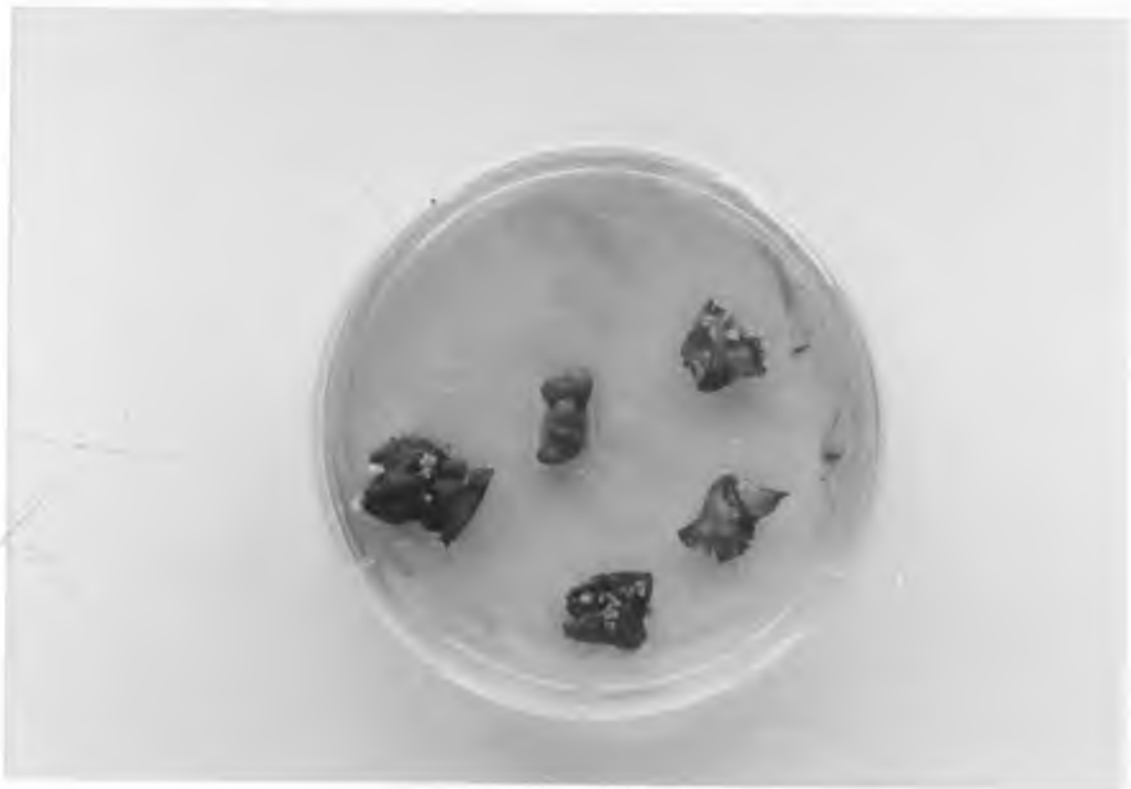


Figure 14: 23-day old leaf discs of var. flavicarpa infected with A. rhizogenes ATCC 15834 showing tumour formation



In contrast, leaf discs of N. tabacum infected with the same strain of A. rhizogenes readily formed roots. The roots were numerous and were observed after 10 days (fig. 15). No tumours were observed on these leaf discs and all the roots arose directly from the leaf disc explants. Five leaf discs were used in this experiment. Two of the leaf discs formed shoots. Leaf discs of P. ligularis and P. subpeltata died within 3 days.

3.9.2 In vivo infection of var. edulis

Tumours were observed growing at all the infected sites of var. edulis, except one, that had been infected with A. tumefaciens A281 in vivo. All the infected sites on the stems started to show evidence of tumour growth after 23 days. The leaves started to show tumour formation after about 30 days. However this growth was on the mid-vein only. No tumour growth occured at the cut leaf apex.

No tumours were observed on the other 3 passion fruit plants infected with A. tumefaciens C58C1(pT1B6S3), A. tumefaciens C58, and A. tumefaciens C58C1. Some of the sites on the plant that had been infected with A. rhizogenes ATCC 15834 started to form tumours after 28 days. A tumour also developed on the mid-vein of the leaf. However, no tumour was observed on the leaf apex. In all the cases no root formation was observed. All the tumours were excised after approximately 4 months. Figure 16 shows tumours incited by A. tumefaciens A281 and figure 17 shows a comparison of the explants infected by the different strains of Agrobacterium.

The tumours were then measured, weighed and surface sterilised using 20% commercial bleach. All the sterilised tumours were incubated on MS medium without hormones. Tables 12 and 13 show the approximate size and weight of the tumours obtained from the different sites of the passion fruit plants.

Tumours incited by A. tumefaciens A281 continued to grow after being incubated on MS medium without hormones. The tumours were subcultured every four weeks when they were subdivided and cultured on fresh MS medium.

Figure 15: 23-day old leaf discs of N. tabacum infected with A. rhizogenes 15834 showing formation of roots

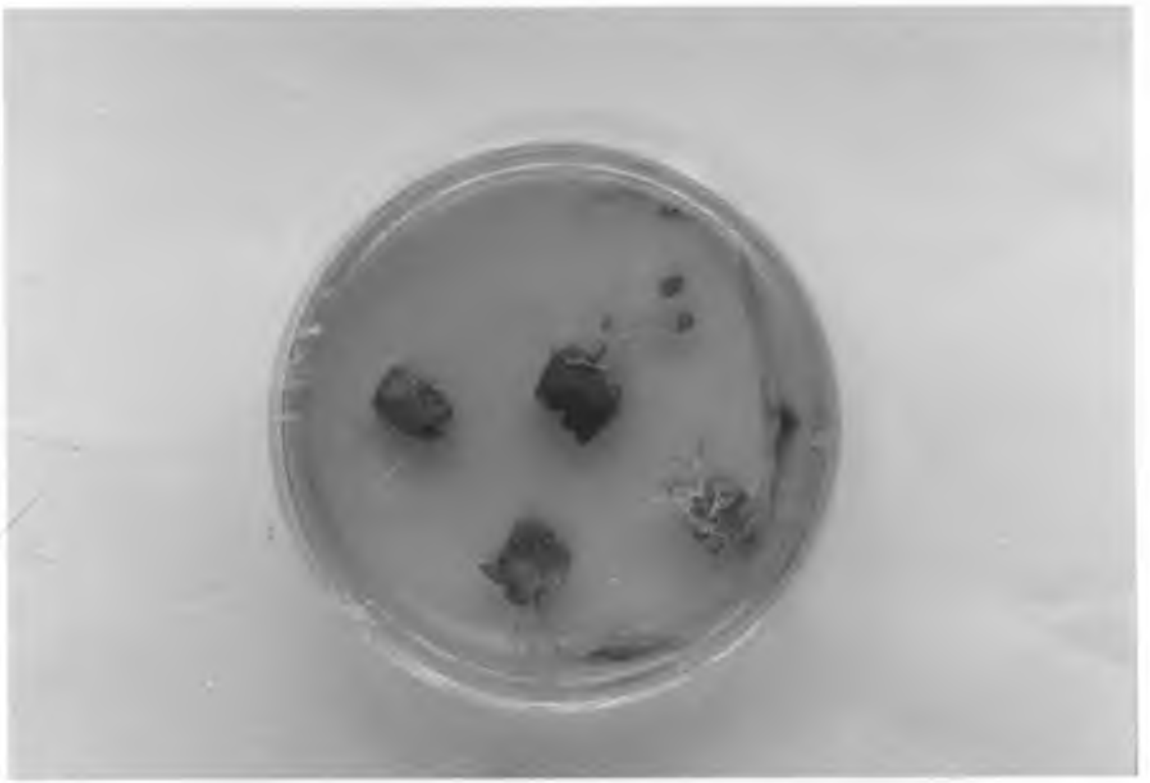


Figure 16: 4-months' old tumours derived from stem and leaf explants of var. edulis infected with A. tumefaciens A281 in vivo

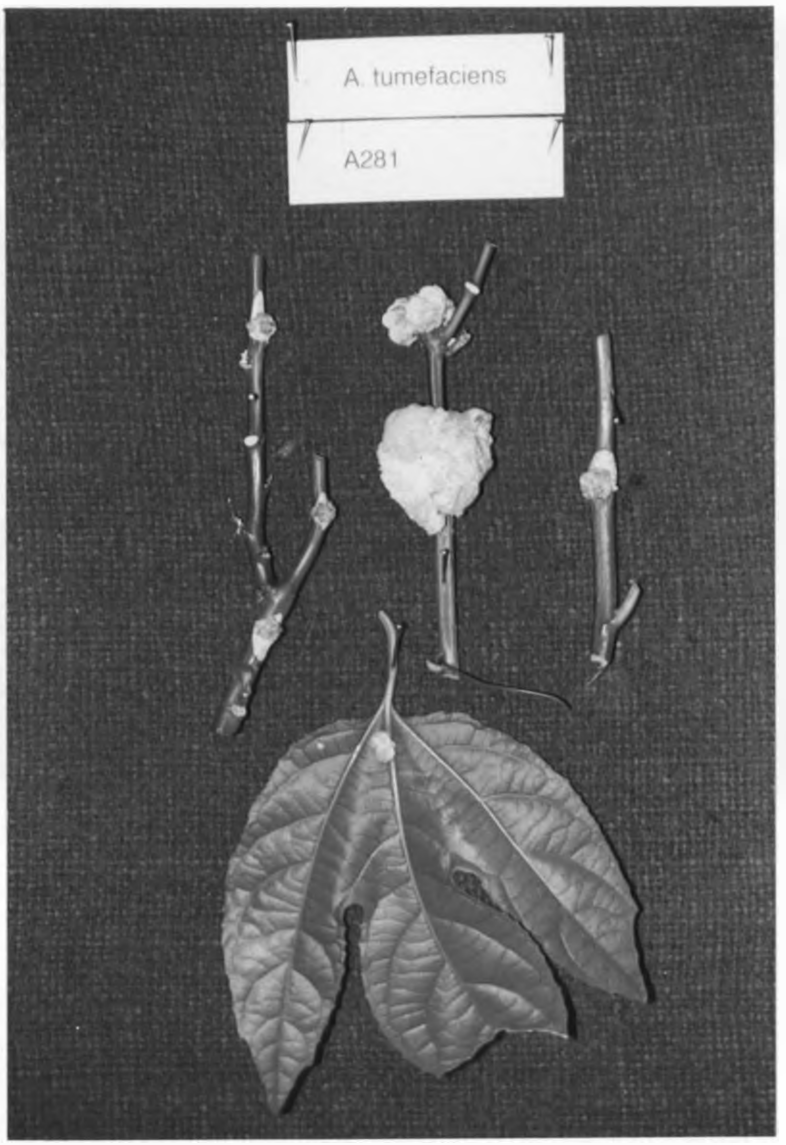


Figure 17: 4-months' old stem fragments of var. edulis infected with different strains of Agrobacterium

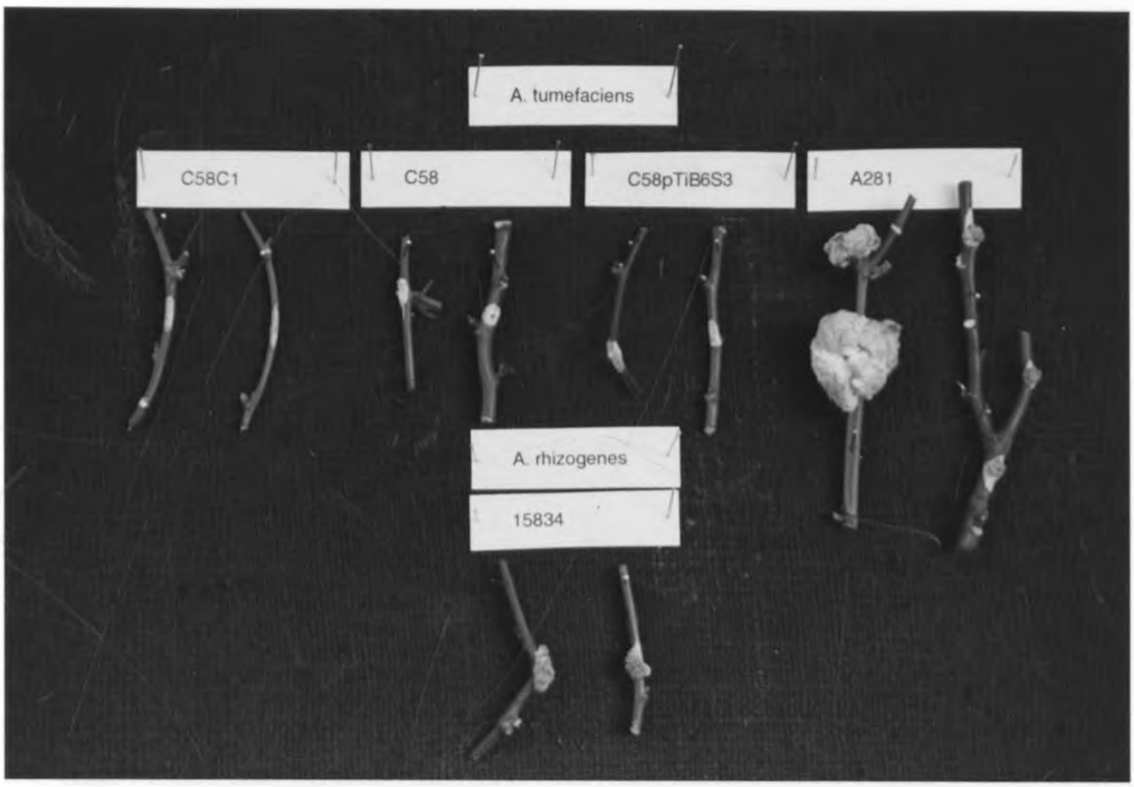


Table 12: Size and weights of tumours incited by A. tumefaciens A281 on P. edulis in vivo

Tumours excised from	Approximate size of tumour (in cm)	Weight of tumour (in mg)
stem apex	1.9 x 2.0 x 0.9	1,920
below stem apex	3.4 x 2.9 x 2.1	7,850
mid stem	0.8 x 0.7 x 0.5	720
base of stem (1)	0.6 x 0.5 x 0.4	450
base of stem (2)	0.9 x 0.6 x 0.5	260
base of stem (3)	0.6 x 0.5 x 0.4	200
mid-vein of leaf	0.7 x 0.5 x 0.4	140

Table 13: Size and weights of tumours incited by A. rhizogenes 15834 on P. edulis in vivo

Tumour excised from	Approximate size of tumour (in cm)	Weight of tumour (in mg)
stem apex	0.3 x 0.4 x 0.2	30
below stem apex	0.3 x 0.35 x 0.3	90
mid stem	1.5 x 1.0 x 0.5	550
base of stem	0.9 x 0.7 x 0.4	250
mid-vein of leaf	0.6 x 0.5x 0.5	90

There were no visible tumours on any of the explants from the passion fruit plant that was infected with A. tumefaciens C58 at the time of incubation on MS medium. However, the explant from the base of the leaf developed a tumour in vitro after 10 days of incubation on MS medium without hormones. This tumour was green in colour. All the explants from the plants that had been infected with A. tumefaciens C58C1(pTiB6S3) and A. tumefaciens C58C1 were lost within 2 weeks of incubation on MS medium due to fungal infection. However, there was no evidence of tumour growth within this period.

All the tumours incited by A. rhizogenes were lost within 2 weeks of incubation on MS medium due to fungal infection. No roots had developed during this period.

3.10 Agropine-mannopine test

This test was done as a preliminary confirmation of transformation. After an electrophoresis was done, the following results were obtained (see fig.18):

1) Callus from passion fruit stem (control). Only one spot was obtained, showing the presence of arginine.

2) Tumour from the stem apex of var. edulis infected with A. tumefaciens A281 in vivo, labelled T1. Two spots were obtained, one for arginine and the other one for agropine.

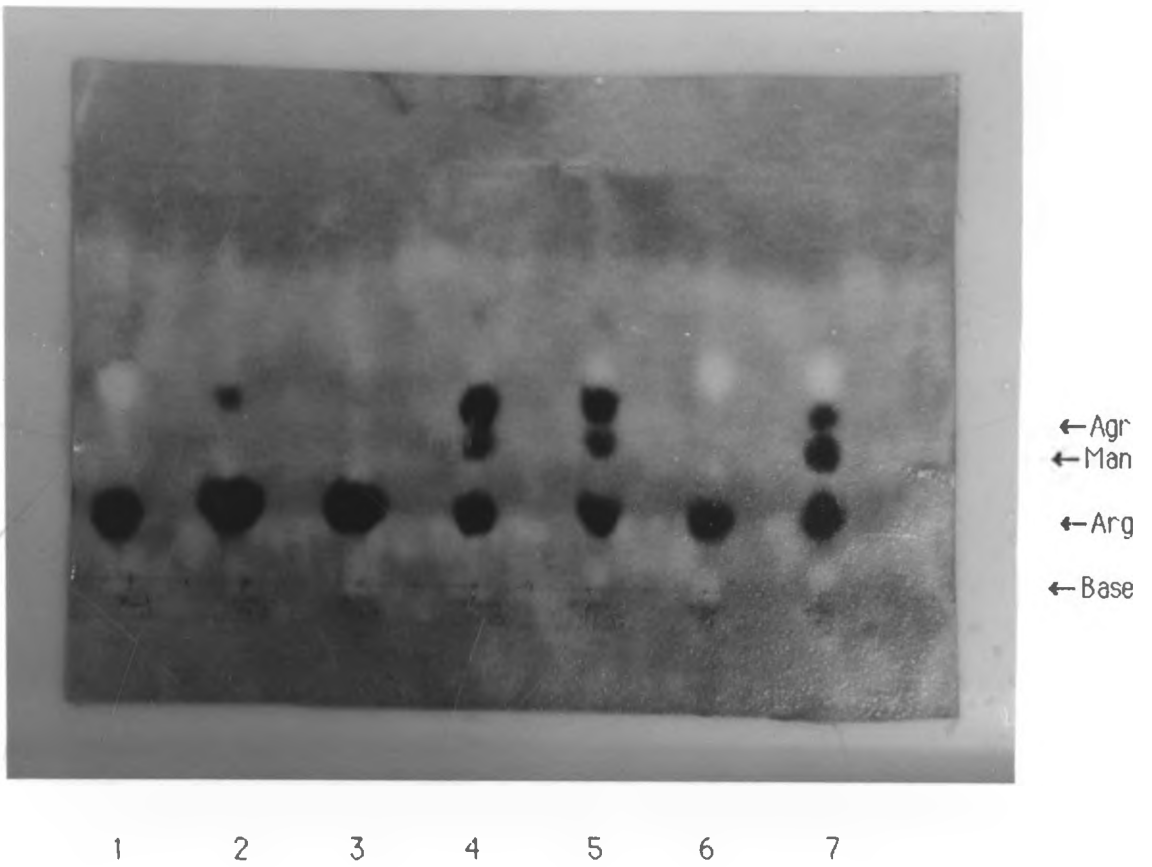
3)Tumour from the youngest part of the stem of var. edulis just below the stem apex incited by A. tumefaciens A281 labelled T2. Only one spot showing presence of arginine was obtained.

4) Tumour from a tobacco leaf disc infected with A. tumefaciens A281 in vitro, labelled T1. Three spots were obtained, one for arginine, and one each for mannopine and agropine, respectively.

5) Tumour from another tobacco leaf disc infected with A. tumefaciens A281, labelled T2. Three spots were obtained as in (4) above.

6) Tumour from a tobacco leaf disc infected with A. tumefaciens C58C1(pTiB6S3) in vitro labelled T1. Only one spot showing presence of arginine was obtained.

Figure 18: A paper electrophoresis run showing presence of arginine, mannopine and agropine, from different tumours and callus tissues of passion fruit and tobacco.



Legend: Agr: Agropine; Man: Mannopine; Arg: Arginine; Base: Baseline
Lane 1: Callus from stem of var edulis
Lane 2: Tumour from var edulis infected with A. tumefaciens A281
Lane 3: Tumour from var edulis infected with A. tumefaciens A281
Lane 4: Tumour from a tobacco leaf disc infected with A. tumefaciens A281
Lane 5: Tumour from a tobacco leaf disc infected with A. tumefaciens A281
Lane 6: Tumour from a tobacco leaf disc infected with A. tumefaciens C58C1(pTiB6S3)
Lane 7: Tumour from a tobacco leaf disc infected with A. tumefaciens C58C1(pTiB6S3)

7) Tumour from a tobacco leaf disc infected with A. tumefaciens C58C1(pTiB6S3) in vitro labelled T2. Three spots showing presence of arginine, mannopine and agropine respectively, were obtained.

CHAPTER FOUR

Discussion

4.1 Germination of seeds

Purseglove (1968) reported that passion fruit seeds can germinate in soil in 2-4 weeks if they are extracted and sown from ripe fruits without removal from the pulp. This was not tested as this method is not suitable for *in vitro* germination of seeds. Therefore, the pulp from all the seeds used in the experiment was always removed. For those seeds sown in soil, no germination was observed until after 21 days. Seeds that had been pre-treated by soaking in water for 48 hours (standard treatment) before sowing gave the best results (table 4). Most of the seeds that had received this pre-treatment germinated during the fourth week. Seeds that had been pre-treated by soaking in 0.1 M hydrochloric acid for 24 hours followed by transfer to water for another 24 hours started germinating after 32 days. This confirms that pre-treatment with water is necessary for dormancy to be broken as reported by various authors (Chapman, 1963; Ministry of Agriculture, 1981)

Kantharajah and Dodd (1990) reported that passion fruit seeds could be germinated on MS medium after approximately 25 days. We were unable to reproduce these results as none of the seeds inoculated *in vitro* germinated. No germination was observed in any of the 50 seeds that had been pre-treated by soaking in water for 48 hours (standard treatment) and then inoculated *in vitro*. Heat-treatment of the seeds is not suitable, none of the seeds that had been boiled in water for two minutes germinated. No germination was observed in any of the 50 seeds inoculated *in vitro* that had been soaked in 0.1 M hydrochloric acid for 24 hours followed by soaking in distilled water for another 24 hours. Although pre-treatment with acid is a form of chemical scarification (Palevitch and Thomas, 1976), this treatment was ineffective when applied to passion fruit seeds. Similarly, pre-treatment of seeds by soaking in potassium nitrate solution yielded negative results despite the fact that such treatment stimulates germination of certain dormant seeds (Palevitch and Thomas, 1976).

This suggests that there could be inhibitory compounds in the seed coat or in the medium that prevented the seeds from germinating. On the other hand, dormancy could have been broken in those seeds sown in soil by leaching of these inhibitory compounds from the seed coat, by abrupt changes in temperature or by the action of fungi and other micro-organisms. As most these factors were not supplied in in vitro conditions these could be the reason why the seeds failed to germinate.

Dormancy in passion fruit seeds is attributed to the tough seed coats which make it difficult for water to reach the embryo. It was confirmed that dormancy could be broken by scarification and this may be enhanced by treatment of the scarified seeds with gibberellic acid. Jones and Stoddart (1977) reported that gibberellic acid stimulates seed germination where dormancy is due to mechanically resistant seed coats, presence of germination inhibitors or incomplete embryo development. The results obtained (table 5) confirm that in vitro seed germination could be inhibited by mechanical resistance of the coat or impermeability of the seed coat to water or oxygen. The cracks or scratches resulting from scarification promote germination by decreasing the resistance of the seed coat to water or oxygen absorption and to embryo expansion (Delvin and Witham, 1983). However, all the young plantlets that germinated from the scarified seeds were abnormal and therefore this approach was abandoned.

Plants of var. edulis grown in the garden of Chiromo campus, University of Nairobi, flowered and set fruits, whilst no flowers nor fruits were observed on var. flavicarpa and the F1 hybrid. This confirms that var. edulis does best at high altitudes while var. flavicarpa and the F1 hybrid are better suited to the tropical lowlands (Samson, 1986; Maluf, 1989).

Rao (1977) reported that immature seeds of various plants can be germinated in vitro. This was not possible in passion fruit. The only response observed when immature seeds of var. edulis were inoculated was formation of callus which was more prominent on media containing 1-3 mg/l NAA and 0.5 mg/l BAP. Most of the calluses observed were either arising

from the seed stalk or from the seed coat. Such tissues proved unsuitable for callus initiation and subsequent regeneration.

Dormancy due to immature embryos may be found in Orchidaceae (Delvin and Witham, 1983). Such dormancy can only be broken by allowing the embryo to complete development within the seeds in an environment favourable to germination, as has been demonstrated in Citrus (Ohta and Frusato, 1957; Gritter, 1986). In passion fruit, seed dormancy may not be due to immature embryos as these failed to germinate when inoculated on MS medium.

4.2 Initiation of callus and shoots from stem explants of var. edulis and var. flavicarpa

A high concentration of auxin relative to cytokinin was found to be necessary for callus initiation from stem explants of var. edulis. No callus or shoots were observed when auxin : cytokinin ratio was 1:3. It was not established why the calluses from stem explants of var. edulis were slow growing. MS medium supplemented with 2 mg/l NAA and 0.5 mg/l BAP gave the best callus growth.

Similar results were obtained from stem explants of var. flavicarpa although exogenous cytokinin was not necessary for callus initiation. Fast growing callus could be induced from the stems using MS medium supplemented with 2-4 mg/l NAA. Failure to subculture callus during the fourth week could lead to death during the sixth week as the dying stem tissue produced phenolics which eventually killed the growing callus.

Culture initiation also depends on the genotype of the plant and the developmental state of the tissue (Murashige, 1974). Cultures range from friable callus consisting of parenchyma-type cells to highly organised masses with some properties of organ primordia. The type of organisation obtained depends primarily on the hormonal constitution of the culture medium, the plant species and the source of the tissue in the plant (Gresshof, 1978).

In that the genetic make up of var. edulis is distinct from that of var. flavicarpa, the response of the stems of the two passion fruit varieties to callus formation might be expected to be and was also distinct. This could be why callus from stem explants of var. edulis differentiated into roots while that from stems of var. flavicarpa did not. The necessary

conditions for the induction of rhizogenesis vary considerably, and the appropriate conditions for root initiation in some species or variety may be completely ineffective in a closely related species or variety (Street, 1977). Some factors involved in rhizogenesis include auxin, carbohydrates, illumination and photoperiod (Gautheret, 1966). In the passion fruit, presence of auxin was found to be an important factor in determining rhizogenesis. In contrast, explants that were inoculated on MS medium supplemented with 2 mg/l BAP failed to form any roots, although they formed a small callus.

In most plants, each leaf has an axillary meristem which has the capacity to develop into a shoot identical to the main shoot (Hussey, 1986). Axillary meristems are inhibited to varying extents, according to the type of plant and how much it naturally branches, by apical dominance. Although the mechanisms of inhibition are complex and involve a number of hormones, the development of the axillary shoot seems to be dependent ultimately on the supply of cytokinin (for a review, see Phillips, 1975). Cytokinin included in the nutrient medium generally has the marked effect of promoting the outgrowth of the axillaries in cultured buds, although its continuous presence may become inhibitory in later stages.

In both varieties of passion fruit, a high concentration of cytokinin relative to auxin was found to be necessary for shoot outgrowth from nodal stem explants. Shoot outgrowth was best on MS medium supplemented with 2 mg/l BAP or 2 mg/l BAP and 0.2 mg/l NAA. Response was poor when 2 mg/l KIN was used as no shoots were observed. The shoots obtained from nodal segments of stem explants grew into apically dominant shoots when subcultured to hormone free MS medium.

In var. flavicarpa, buds were observed growing from one cut end of the stem segments (without nodes) inoculated on MS medium supplemented with 2 mg/l BAP. These buds failed to develop into proper shoots, with definite stems and leaves, after being cut off from the initiating explants and subsequently transferred to medium with 2 mg/l BAP. This suggests that stem explants of var. flavicarpa have totipotent cells capable of regenerating shoots and

further work is needed to establish whether such shoots could be made to develop stems and leaves.

Hussey (1986) reported that adventitious shoots or embryos may be formed from callus if the concentrations of hormones, especially auxin is lowered. Although callus may be obtained from virtually any plant species, it can only be regenerated in some cases. Such failure to regenerate shoots was observed in passion fruit callus derived from the stems. Perhaps such calluses had a high proportion of polyploid or aneuploid cells, which often reduce the ability to form shoots (Smith and Street, 1974). Only certain cells in callus are totipotent, and unless these continue to divide at the average rate for the callus as a whole they will in time be diluted out (Hussey, 1986).

4.3 Initiation of callus, roots and shoots from leaf discs

In their classical experiments with cultured stem tissue of tobacco, Skoog and Miller (1957) showed that different types of organogenesis can be obtained by varying the concentrations of auxins and cytokinins in the culture medium. When the concentrations of cytokinin are high relative to auxin, shoots are induced ; when the concentration of cytokinin is low relative to auxin, roots are induced and at intermediate concentrations, the tissues grow as unorganised callus. This basic approach has been used to regenerate a wide variety of dicotyledonous plants (Murashige, 1974; Narayanaswamy, 1977). In contrast, monocotyledonous species do not show a pronounced response to cytokinins and require high concentrations of potent auxins such as 2,4-D to achieve changes in the development of cultured tissues (Gresshof, 1978).

The induction of adventitious shoots directly from organ explants begins with cell divisions either in fully differentiated cells such as epidermis or cortex parenchyma or in more meristematic tissue such as the vascular cambium or root pericycle (Hussey, 1986). Cytokinins influence root growth, cell division, cellular enlargement and differentiation, lateral bud development and shoot formation (Delvin and Witham, 1983). When leaf discs of var. flavicarpa were inoculated on MS medium supplemented with 2 mg/l BAP or 2 mg/l BAP

and 0.5 mg/l kinetin, shoots were induced within 4 weeks. The leaf discs would enlarge in size prior to shoot formation, probably due to presence of cytokinin. Cytokinins also induce cell enlargement, an effect usually associated with IAA or gibberellins (Delvin and Witham, 1983). Although some callus formed at the edges of the mid-vein there was no evidence that the shoots were arising from the callus. Hussey (1986) reported that there is mostly no sharp dividing line between the formation of adventitious shoots directly from the parent tissue and the regeneration of shoots from intermediate callus. In the case of var. flavicarpa, the shoot regeneration observed probably arose directly on organ explants as some shoots were observed on leaf discs that were not callusing.

Little is known about how hormones evoke a particular pattern of morphogenesis or the mechanism of induction at the cell and molecular level (Thorpe, 1980). In dicotyledonous species, for example, different types of development can be obtained by treating the same cloned line of cells with different combinations of hormones (Meins, 1986). Leaf discs of var. flavicarpa inoculated on media with 2 mg/l NAA or 2 mg/l NAA and 0.5 mg/l kinetin formed callus and roots within 4 weeks. Those on medium with 2 mg/l NAA, 2 mg/l IAA and 0.4 mg/l kinetin formed only callus while those on medium with 2 mg/l BAP or 2 mg/l BAP and 0.5 mg/l kinetin formed a little callus and shoots, within 4 weeks. In similar experiments leaf discs of var. edulis did not form any callus, roots or shoots.

The initiation of proliferating cultures from tissue explants involves profound changes in developmental state: the basic architecture of the tissue is altered, certain specialised cell types are lost, new cell types arise and normally quiescent cells begin to divide (Gautheret, 1966). Many plant tissues show an absolute requirement for exogenous auxin and cytokinin for sustained proliferation on an otherwise complete medium containing sources of carbon and nitrogen, inorganic salts and vitamins (Gresshof, 1978). Thus, hormonal constitution of the culture medium is a critical factor in initiating long term cultures. In passion fruit, leaf discs of var. flavicarpa showed an absolute requirement for auxin in order to form callus and roots, and cytokinin in order to form shoots. However, leaf discs of var. edulis failed to form callus,

roots or shoots when various concentrations of auxin and cytokinin were used (see table 9). Further work has to be done to establish the reason behind the failure of the leaf discs to form any callus, roots or shoots.

Hussey (1986) reported that although a number of plants can root spontaneously in vitro, shoots of most plants multiplied in vitro lack a root system. Rooting can be achieved either by subculturing to medium lacking cytokinin, with or without a rooting hormone. All cytokinins inhibit rooting and BAP does so particularly strongly, even after transfer to cytokinin free medium. Many species require the stronger auxins IBA or NAA to stimulate ro formation although IAA can also induce rooting. NAA usually gives rise to short thick roots which may have the advantage (as in the case of pear; Lane, 1979) of being better able to withstand accidental damage during planting out.

The shoots obtained from leaf discs of var. flavicarpa could be induced to root when transferred to MS medium supplemented with: 0.1, 0.5, or 1, mg/l NAA. Roots started to appear after 10 days. Kantharajah and Dodd (1990) were unable to induce roots on the shoo obtained from nodal segments of var. edulis using IBA. In var. flavicarpa, no roots were observed when the shoots were transferred to MS medium supplemented with 0.5 mg/l IBA. However, shoots obtained from leaf discs of P. alato-caerulea were induced to root when transferred to medium containing 0.5 mg/l IBA (Muralidhar and Mehta, 1982). This sugges that the inability of IBA to induce rooting in passion fruit is only unique to this species, but does not apply to the genus Passiflora.

When leaf discs of var. flavicarpa were cultured on MS medium supplemented with 2 mg/l BAP or 2 mg/l BAP and 0.5 mg/l KIN, shoots were observed in both cases after about 2 days. This suggests that the cytokinin supplement is not absolutely necessary in order to induce shoots. Leaf discs of P. alato-caerulea cultured on MS medium with 2 mg/l BAP and 0 mg/l KIN formed visible shoots on day 15 (Desai and Mehta, 1985). In var. flavicarpa, bud started to appear on the leaf discs cultured on medium with 2 mg/l BAP after 13 days. However, definite shoots with stems and leaves appeared after about 20 days.

No more shoots were observed when leaf discs of var. flavicarpa that had already formed shoots were subcultured to the same medium (containing 2 mg/l BAP or 2 mg/l BAP and 0.5 mg/l kinetin), after the removal of the shoots that had formed in the first instance. This suggests that the morphogenetic competence of the cells gradually declines during prolonged culture or that only a certain number of cells are totipotent. However, some of the leaf discs that had not formed shoots by day 28 and were subcultured to the same medium eventually formed shoots. In this case, it is possible that the competent cells of such leaf discs had already reacted to the growth regulators but were not yet committed to a particular developmental fate (Halperin, 1969; Henshaw et al., 1982). A second treatment with the same growth regulators therefore started the cells on a specific developmental pathway, hence the formation of shoots.

Totipotent cells have the potential to form the full range of plant organs. However, under a particular set of experimental conditions, specific types of organogenesis occur to the exclusion of other types. This suggests that cultured cells vary in their competence for morphogenesis and responsiveness to inducers. Distinct states of competence for root formation and shoot formation can be recognised by their stability and conditions for induction (Gresshof, 1978). In some cases, these conditions are already in the plant and tissues from different parts of the same plant form different organs under comparable conditions in culture (Chaturvedi and Mitra, 1975). In other cases, distinct states of morphogenetic competence can be induced in culture. In passion fruit, leaf discs of var. flavicarpa could be induced to form callus, callus and roots, or shoots depending upon the concentrations of auxin and cytokinin in the culture medium (see table 10).

In P. suberosa, shoot formation required only a 1-day exposure to BAP but flowering required a 3-day exposure (Scorza and Janick, 1980). Flowering occurred in as few as 21 days and cytokinin was found to be an absolute requirement for flowering. BAP induced flowering from stem segments, leaf discs and tendril segments. In var. flavicarpa, cytokinin was an absolute requirement for shoot formation only as no other organs were observed.

Desai and Mehta (1985) reported that leaf discs of P. alato-caerulea cultured on medium with 2 mg/l NAA and 0.5 mg/l kinetin formed callus after about 9 days and roots formed after 23 days. Similar results were obtained from leaf discs of var. flavicarpa cultured on the same medium although callus began to appear on day 4 while roots started to appear on day 13. Leaf discs of var. flavicarpa cultured on medium with 2 mg/l NAA also formed callus and roots, suggesting that kinetin is not absolutely necessary for such a response to be invoked. The roots induced on media with NAA were thicker than normal passion fruit roots.

Scorza and Janick (1979) and Kantharajah and Dodd (1990) reported that leaf discs of var. edulis can form callus and root initials when cultured on MS medium supplemented with various combinations of auxin and cytokinin, for example, 1 mg/l NAA and 1 mg/l BAP. However, these experiments could not be reproduced. Leaf discs of var. edulis failed to form callus, roots or shoots. Failure to obtain regeneration suggests that there could be a lot of genetic variation among the purple passion fruit that has not been studied. Most of the leaf discs used started to turn brown during the sixth week and death would eventually follow. Perhaps the various concentrations of auxin and cytokinin used were unable to stimulate cell division amongst the cells that were in contact with the medium.

4.4 Infection with Agrobacterium

Results obtained show that A. tumefaciens causes neoplastic transformation, called crown gall, on wounded passion fruit tissue. The molecular basis of this transformation is the transfer and stable integration of the T-DNA (Chilton et al., 1977). This study also confirmed that A. tumefaciens A281 is a hypervirulent strain as the tumours incited by this bacterial strain were the first to appear, both in passion fruit and in tobacco. The tumours were able to grow on hormone free medium, free of the inciting bacterium. For crown gall tumorigenesis the continuous presence of the bacterium is not necessary because the bacterium transfers the phytohormones biosynthetic genes to the plant (Gelvin, 1990). Tumours incited on tobacco leaf discs by A. tumefaciens C58C1(pTiB6S3) also continued to grow on hormone free medium.

It was not established why the nopaline strain C58 and the octopine strain C58C1 (pTiB6S3) were unable to induce tumours on passion fruit plants infected in vivo. However, the fact that the leaf from the plant infected with the nopaline strain C58 in vivo eventually formed a tumour when incubated on MS medium shows that passion fruit could also be susceptible to infection by strain C58. No tumours were observed on the plants infected with strain C58C1 because this bacterial strain has been cured of its Ti plasmid (Van Larebeke et al., 1975), and this was a control experiment.

It was not established why most leaf discs of passion fruit, those that had formed tumours and those that had not, started to turn brown after 20 days of culture and eventually died. The nopaline strain C58 induced shoots on some of the tobacco leaf discs in addition to tumours. This confirmed an observation by De Greve et al. (1982), that normal and fertile plants which still contain and express T-DNA sequences, can exceptionally be regenerated from tobacco crown gall tumour cells.

The passion fruit plant infected with A. rhizogenes ATCC 15834 in vivo formed tumours but no roots. This confirmed the observation by De Cleene and De Ley (1981) that A. rhizogenes can also induce undifferentiated tumours, when infected on some plants. Tumours were also observed on the leaf discs of var. flavicarpa and the F1 hybrid infected with the same bacterial strain of A. rhizogenes in vitro. Only one root was observed arising from one of the tumours. In tobacco, however, numerous roots were formed directly from the leaf disc.

In general, var. flavicarpa and the F1 hybrid were more susceptible to Agrobacterium infection than var. edulis. This could be attributed to the differences in their genetic make up.

4.5 Agropine-mannopine test

Opines were not detected in the callus tissue derived from stem explants of passion fruit. This shows that such tissues were not transformed. Furthermore, no arginine metabolism had taken place in such callus tissue as this can also lead to formation of opines

(Christou et al., 1986). The fact that this callus could only proliferate on medium with growth regulators was further evidence that the tissue was not transformed.

Agropine was detected in one of the tumours incited on passion fruit by A. tumefaciens A281. Although tumours incited by this bacterial strain can produce leucinopine, mannopine, mannopinic acid, agropinic acid and agrocinopines C and D, in addition to agropine (Hood et al., 1984), only presence of agropine or mannopine were tested for. No mannopine was detected in any of the tumour tissues from passion fruit. However, all the tumour tissues from the tobacco leaf discs infected with A. tumefaciens A281 showed presence of mannopine and agropine.

Only one tumour from tobacco leaf discs infected with the octopine strain C58C1 (pTiB6S3) showed presence of mannopine and agropine while the other tumour did not. The TR region that codes for genes responsible for synthesis of agropine and mannopine is not always present. During tumour formation, the TR region may not be transferred to the host or may fail to integrate. It is possible that the tumour which lacked opines did not have this TR region. As the tumours were tested after only 2 days of incubation on MS medium, it is unlikely that the TR region was lost as a result of subculture, although Salomon et al. (1984) reported that mannopine and agropine can no longer be detected after several months of subculture.

Presence of opines in passion fruit tumour tissue is only a preliminary indication of transformation by Agrobacterium. Further work has to be done in order to establish whether transformation has taken place. However, the appearance of tumours able to grow on hormone free medium and the presence of opines is a good indication of transformation.

4.5 Conclusion

One striking difference observed during the study, is the response of the two passion fruit varieties to tissue culture. The fact that a complete regeneration method has been described for the yellow passion fruit (var. flavicarpa) opens the possibility for further work to be done as this crop is of economic importance to Kenya. Failure of the purple passion fruit (var. edulis) to elicit the desired response does not necessarily mean that the plant is

recalcitrant to tissue culture. What needs to be done is to identify a suitable starting material and an appropriate concentration of hormones, as the experience with var. flavicarpa has shown that the starting material is of importance if complete regeneration has to be achieved.

The response of both varieties to infection with Agrobacterium was encouraging. Results obtained showed that passion fruit is susceptible to infection by Agrobacterium tumefaciens A281 and Agrobacterium rhizogenes ATCC 15834.

4.6 Recommendations

Future work should include a tissue culture method that can be used for multiplication of var. edulis as has been described for var. flavicarpa. Failure of the leaf discs of var. edulis to form callus, roots or shoots when inoculated on medium supplemented with various concentrations of auxin and cytokinin needs further investigation. In addition failure of IBA to induce rooting could also be investigated.

Further work could also be done to establish why regenerated buds that were growing from one cut end of the stems of var. flavicarpa inoculated on medium supplemented with 2 mg/l BAP failed to develop shoots.

Attempts should be made to return plantlets of var. flavicarpa back to in vivo conditions and compare them with plants germinated from seeds.

This study describes a tissue culture system that can be used for multiplication of interesting clones of the yellow passion fruit. The system could also be used to isolate somaclonal variants. Genetic traits that could be studied include wilt resistance, which is controlled by a single locus.

Further work could also be done to see whether passion fruit can be improved by genetic engineering using the Agrobacterium system, especially with regard to its resistance to woodiness disease which is caused by passion fruit woodiness virus.

Literature Cited

Akamine, E. K., Aragaki, M., Beumont, J. H., Bowers, F. A. I., Hamilton, R. A., Nishida, T., Sherman, G. D., Shoot, K., Storey, W. B., Martinez, A. P, Yee, W. Y. T., Shaw, T. N. (1974). Passion fruit culture in Hawaii, Cooperative Extension Service, University of Hawaii, Circular 345.

Akamine, E. K. and Girolami, G. (1959). Pollination and fruit set in the yellow passion fruit. Hawaii Agric. Expt. Stat. Tech. Bul. 39: 44.

Akiyoshi, D. E., Klee, H., Amasino, R. M., Nester, E. W., Gordon, M. P. (1984). T-DNA of Agrobacterium tumefaciens encodes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci. USA 81: 5994-5998.

Bailey, L. H. (1973). Manual of Cultivated Plants, pp. 689-691. The Macmillan Company, New York.

Beiderbeck R. (1973). Wurzelinduktion an Blättern von Kalanchoë daigremontiana durch Agrobacterium rhizogenes und der Einfluß von Kinetin auf diesen Prozess. Z. Pflanzenphysiol. 68: 460-467.

Binns, A. N. and Thomashow, M. F. (1988). Cell biology of Agrobacterium infection and transformation of plants. Ann. Rev. Microbiol. 42: 575-606.

Boulton, G. W., Nester, E. W., Gordon, M. P. (1986). Plant phenolic compounds induce expression of Agrobacterium tumefaciens loci needed for virulence. Science, 232: 983-985.

Bytebier, B., Deboeck, F., De Greve, H., Van Montagu, M., Hernalsteens, J.-P. (1987). T-DNA organization in tumour cultures and transgenic plants of the monocotyledon Asparagus officinalis. Proc. Natl. Acad. Sci. U.S.A. 84: 5345-5349.

Chapman, T. (1963). Passion fruit growing in Kenya. Econ. Bot. 17: 165-168.

Chaturvedi, H. C. and Mitra, G. C. (1975). A shift in morphogenetic pattern in Citrus callus tissues during prolonged culture. Ann. Bot. 390: 683-687.

Chilton, M-D., Drummond, H. J., Merlo, D. J., Sciaky, D., Montoya, A. L., Gordon, M. P., Nester, E. W. (1977). Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 11: 263-271.

Chilton, M-D., Tepfer, D. A., Petit, A., David, C., Cass-Delbart, F., Tempé, J. (1982). Agrobacterium rhizogenes inserts T-DNA into the genomes of of the host plant root cells. Nature 295: 432-434.

Chilton, W. S., Tempé, J., Matzke, M., Chilton, M.-D. (1984). Succinamopine: a crown gall opine. J. Bacteriol. 157: 357-362.

Christou, P., Platt, S. G., Ackerman, M. C. (1986). Opine synthesis in wild-type plant tissue. Plant Physiol. 82: 218-221.

Cox, J. E. and Kiely, T. B. (1961). Fusarium resistant rootstocks of passion fruit vines. Agric. Gaz. New South Wales 72: 314-318.

David C., Chilton, M-D., Tempé, J. (1984). Conservation of T-DNA in plants regenerated from hairy root cultures. Bio/Technology 2: 73-76.

De Cleene, M. (1985). The susceptibility of monocotyledons to Agrobacterium tumefaciens. Phytopath. Z. 113: 81-89.

De Cleene, M. and De Ley, J. (1976). The host range of crown gall. Bot. Rev. 42: 389-466.

De Cleene, M. and De Ley, J. (1981). The host range of infectious hairy-root. Bot. Rev. 47: 147-194.

Degener, O. (1933). Flora Hawaiiensis Book 1. Honululu.

De Greve, H., Leemans, J., Hernalsteens, J. P., Thia-Toong, L., De Beuckleer, M., Willmitzer, L., Otten, L., Van Montagu, M., Schell, J. (1982). Regeneration of normal and fertile plants that expreess octopine synthase, from tobacco crown galls after deletion of tumour-controlling functions. Nature 300: 752-755.

Dell, B. (1980). Structure and function of the strophiolar plug in seeds of Albizia lophantha. Am. J. Bot. 67: 556-563.

Delvin, R. M. and Witham, F. H. (1983). Plant Physiology. CBS publishers and distributors, New Delhi.

Depicker, A., De Wilde, M., De Vos, G., De Vos, R., Van Montagu, M., Schell, J. (1980). Molecular cloning of overlapping segments of the nopaline Ti-plasmid pTi C58 as a means to restriction endonuclease mapping. Plasmid 3: 193-211.

Desai, H. V. and Mehta, A. R. (1985). Changes in polyamine levels during shoot formation, root formation and callus induction in cultured Passiflora leaf discs. J. Plant Physiol. 119: 45-53.

Dixon, R.A. (1985). Plant Cell Culture: a practical approach, IRL Press, Oxford.

Dodds, J. H. and Roberts, L.W. (1982). Experiments in Plant Tissue Culture. Cambridge University Press, London.

Evans, D. A. and Sharp, W. R. (1983). Single gene mutations in tomato plants regenerated from tissue culture. Science 221: 949-951.

Evans, D. A. and Sharp, W. R. (1986). Applications of somaclonal variation. Bio/Technology 4: 528-532.

Firmin, J. L. and Fenwick, G. R. (1978). Agropine-a major new plasmid-determined metabolite in crown gall tumours. Nature 276: 842-844.

Gautheret, R. J. (1966). Factors affecting differentiation of plant tissues In vitro. In: Cell Differentiation and Morphogenesis (ed. W. Beerman) pp. 55-95. Amsterdam-North Holland.

Gelvin, S. B. (1990). Crown gall disease and hairy root disease: a sledgehammer and a tackhammer. Plant Physiol. 92: 281-285.

Genetello, Ch., Van Larebeke, N., Holsters, M., Depicker, A., Van Montagu, M., Schell, J. (1977). Ti plasmid of Agrobacterium as conjugative plasmids. Nature 265: 561-563.

George, E. F. and Sherrington, P. D. (1984). Plant Propagation by Tissue Culture, Exegetics Ltd., Basingstoke, England.

Gilmour, J. G. (1983). A guide to granadilla growing in Zimbabwe. Zimbabwe Agricultural Journal 80: 83-92.

Gresshoff, P. M. (1978). Phytohormones and growth and differentiation of cells and tissues of cells cultured in vitro. In: Phytohormones and Related Compounds- A Comprehensive Treatise (eds. Letham, D. S., Goodwin, P. B. and Higgins, T. J. V.), vol. 2, pp. 1-29. Elsevier. Amsterdam, North Holland.

Grimsley, N., Hohn, B., Ramos, C., Kado, C., Rogowsky, P. (1989). DNA transfer from Agrobacterium to Zea mays or Brassica by agroinfection is dependent on bacterial virulence functions. Mol. Gen. Genet. 217: 309.

Gritter, F. G. (1986). Citrus embryogenesis in vitro: culture initiation, plant regeneration and phenotypic characterization. Dissert. Abstr. Intern. 3 (Science and Engineering) 47: 13578.

Halperin, W. (1969). Morphogenesis in cell cultures. Ann. Rev. Plant. Physiol. 20: 395-417.

Henshaw, G. G., O'Hara, J. F., Webb, K. J. (1982). Morphogenetic studies in plant tissue cultures. Symp. Brit. Soc. Biol. 4: 231-251.

Herrera-Estrella, L. and Simpson, J. (1988). Foreign gene expression in plants. In: Plant Molecular Biology: a practical approach (ed. Shaw, C H.) pp.131-138. IRL Press, Oxford.

Hoagland, D. R. and Arnon, D. I. (1938). The water-culture method for growing plants without soil. Univ. Calif. Coll. Agric. Exp. Stat. Circ. 347.

Hood, E. E., Jen, G., Kayes, L., Kramer, J., Fraley, R. T., Chilton, M. (1984). Restriction endonuclease map of pTiBo542, a potential Ti plasmid vector for genetic enginneering of plants. Bio/Technology 2: 701-709.

Hooykaas, P. J. J., Hofker, M., den Dulk-Ras, H., Schilperoort, R, A. (1984). A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri

plasmid by complementation analysis of Agrobacterium tumefaciens mutants. Plasmid 11: 195-205.

Horsch R. B., Fry, J. E., Hoffman, N. L., Wallroth, M., Eichholtz, D., Rogers, S. G., Fraley, R. T. (1985). A simple and general method for transferring genes into plants. Science 227: 1229-1231.

Horticultural Crops Development Authority (1992). Personnal communication.

Hussey, G. (1986). Vegetative propagation of plants by tissue culture. In: Plant Cell Culture Technology (ed. Yeoman. M. M.) pp. 29-66. Blackwell Scientific Publications, London.

Jin, S., Komari, T., Gordon, P. M., and Nester, W.E., (1987). Genes responsible for supervirulence phenotype of Agrobacterium tumefaciens A281. J. Bacteriol. 169: 4417-4428.

Jones, R. L. and Stoddart, J. L. (1977). Gibberellins and seed germination. In: The Physiology and Biochemistry of Seed Dormancy and Germination (ed. Khan, A. A.) pp. 77-104. Elsevier Biomedical Press. Amsterdam, North-Holland.

Kado, C. I. (1991). Molecular mechanisms of crown gall tumorigenesis. Critical Reviews in Plant Sciences 10: 1-32.

Kantharajah, A.S. and Dodd, W.A. (1990). In vitro micropopagation of Passiflora edulis (purple passion fruit). Ann. Bot. 65: 337-339.

Karp, A. (1989). Can genetic instability be controlled in plant tissue culture. International Association for Plant Tissue Culture Newsletter 58: 2-11.

Khan, A. A. (1971). Cytokinins: permissive role in seed germination. Science 171: 853-859.

Klee, H. Horsch, R., Rogers, S. (1987). Agrobacterium-mediated plant transformation and its further plant applications to plant biology. Ann. Rev. Plant Physiol. 38: 467-486.

Knight, R. J. and Winters, H. F. (1963). Effects of selfing and crossing in the yellow passion fruit. Proc. Florida State Hort. Soc. 76: 345-347.

Krikorian, A. D. (1982). Cloning higher plants from aseptically cultured tissues and cells. Biol. Rev. Cambridge 57: 151-218.

Kuhne, F. A. (1968). Cultivation of Granadillas (1). Farming in South Africa. 307. 29-32.

Lane, W. D. (1979). Regeneration of pear plants from shoot meristem tips. Plant Sci. Lett. 16: 337-342.

Larkin, P. J. and Scowcroft, W. R. (1981). Somaclonal variation: a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60: 197-214.

Leemans, J., Shaw, C., Deblaere, R., De Greve, H., Hernalsteens, J. P., Van Montagu, M., Schell, J. (1981). Site-specific mutagenesis of Agrobacterium Ti plasmids and transfer of genes to plant cells. J. Mol. Appl. Genet. 1: 149-164.

Leifert, C. and Waites, W. M. (1990). Contaminants of plant tissue cultures. International Association for Plant Tissue Culture Newsletter 60: 2-13.

Lippman, D. (1978). Cultivation of Passiflora edulis Sims. General information on passion fruit growing in Kenya. GTZ Report 62.

Maluf, W. R., Silva, J. R., Grattapaglia, D., Toma-Braghini, M., Corte, R. D., Machado, M. A., Caldas, L. S. (1989). Genetic gains via clonal selection in passion fruit, Passiflora edulis Sims. Brazil. J. Genetics 12: 833-841.

Martin, F. W. and Nakasone, H.Y. (1970). The edible species of Passiflora. Econ. Bot. 24: 330-340.

Meins, F. (1986). Determination and morphogenetic competence in plant tissue culture. In: Plant Cell Culture Technology (ed. M. M. Yeoman). Blackwell Scientific Publications, London.

Messens, E., Dekeyser,R., Stachel, S. E. (1990). A non-transformed Triticum monococcum monocotyledonous culture produces the potent Agrobacterium vir-inducing compound ethyl ferulate. Proc. Natl. Acad. Sci. U.S.A. 87: 4368-4372.

Miller, J. H. (1972). Experiments in Molecular Genetics. Cold Spring Harbour, New York.

Ministry of Agriculture (1981). Passion Fruit (Passiflora spp.). In: Major Crops: Technical Handbook, pp. 37-40. Agricultural Information Centre, Nairobi.

Montaldi, E. R. (1972). Kinetin induction of bud differentiation on roots of entire plants. Z. Pflanzenphysiol. 67: 43-44.

Moran Robles, M. J. (1978). Multiplication vegetative, in vitro, des bourgeons axillaires de Passiflora edulis var. flavicarpa Degener et de P. mollissima Bailey. Fruits 33: 693-699.

Morel, G. (1956). Metabolisme de l'arginine par les tissus de crown gall de topinambour. Bull. Soc. Fr. Physiol. Veg. 2: 75.

Muralidhar, C. E. and Mehta, A. R. (1982). Clonal propagation of three ornamental plants through tissue culture methods. In: Plant Tissue Culture 1982. Proc. 5 th. Int. Cong. Plant. Tiss. Cell Cult., Japan. (ed. Fujiwara, A.) pp. 693-694. Jap. Assoc. Plant Tissue Culture, Tokyo.

Muralidhar, C. E. and Mehta, A. R. (1986). In vitro propagation of Passiflora alato-caerulea from cultured leaf discs. Hort. Science 21: 804.

Murashige, T. and Skoog, F. (1962). A medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Murashige, T. (1974). Plant propagation through tissue culture. Ann. Rev. Plant Physiol. 25: 135-165.

Nakayama, W. (1966). In vitro culture of tissues of P. caerulea. Ann. Rev. Plant Phsiol. 25: 135-165.

Narayanaswamy, S. (1977). Regeneration of plants from tissue cultures. In: Plant Cell, Tissue and Organ Culture (eds. Reinhert, J. and Bajaj, Y. P. S.) pp. 179-248. Springer-Verlag, Berlin.

Nattrass, R. M. (1939). A preliminary note of the woodiness disease of passion fruit in Kenya. E. Afric. Agric. J. 2: 130-133.

Nattrass, R. M. (1940). Further notes on the woodiness disease of the passion fruit in Kenya. E. Afric. Agric. J. 3: 54.

Nishida, T. (1963). Ecology of the pollinators of passion fruits. Hawaii Agric. Exp. Stat. Bul. 55: 38.

Ohta, Y. and Frusato, K. (1957). Embryo culture in Citrus. Report of the Kihara Institute for Biological Research. 8: 49-54.

Palevitch, D. and Thomas, T. H. (1976). Enhancement by low pH of gibberellin effects on dormant celery seeds and embryoless half-seeds of barley. Physiol. Plant. 37: 247-252.

Petit, A., Tempe, J., Kerr, A., Holsters, M., Van Montagu, M., Schell, J. (1978). Substrate induction of conjugative activity of Agrobacterium tumefaciens Ti plasmids. Nature 271: 570-571.

Philips, I. D. J. (1975). Apical dominance. Ann. Rev. Plant Physiol. 26: 341-367.

Pope, W. T. (1935). The edible passion fruit in Hawaii. Hawaii Agric. Exp. Sta. Bul. 74: 22.

Pruthi, J. S. (1963). Physiology, chemistry and technology of passion fruit. Advan. Food Res. 12: 203-282.

Purseglove, J. W. (1968). Tropical Crops, Dicotyledons 2. pp. 420-429. Longman Green Company, London.

Rao, A. N. (1977). Tissue culture in orchid industry. In: Plant Cell, Tissue and Organ Culture (eds. Reinhert, J.and .Bajaj, Y. P .S). Springer-Verlag, Berlin.

Reinhert, J. and Bajaj, Y. P. S. (1977). Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, pp. 87-92. Springer-Verlag, Berlin.

Sahi, S. V., Chilton, M.-D., Chilton, W. S. (1990). Corn metabolites affect growth and virulence of Agrobacterium tumefaciens. Proc. Natl. Acad. Sci. U.S.A. 87: 3879-3883.

Salomon, F., Deblaere, R., Leemans, J., Hernalsteens, J.-P., Van Montagu, M., Schell, J. (1984). Genetic identification of functions of TR-DNA transcripts on octopine crown galls. Embo J. 3: 141-146.

Samson, J. A. (1986). Tropical Fruits.pp. 291-294. Longman Publishers Ltd. Singapore.

Scorza, R. and Janick, J. (1979). Tissue culture in Passiflora. 24 th Annual Congress of the American Soc. Hort. Sci. Tropical Region-Mayaguez, Puerto Rico, pp. 179-183.

Scorza, R. and Janick, J. (1980). In vitro flowering of Passiflora suberosa L.. J. Amer. Soc. Hort. Sci. 105: 892-897.

Skoog, F. and Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues in vitro. In: The Biological Action of Growth Substances (ed. Porter, H. K), pp. 118-131. Symposia of the Society for Experimental Biology No. 11, Cambridge University Press, Cambridge.

Smith, S. M. and Street, H. E. (1974). The decline of embryogenic potential as callus and suspension cultures of carrot (Daucus carota L.) are serially subcultured. Ann. Bot. 38: 223-241.

Stachel, S. E. and Zambrisky, P. C. (1986). VirA and virG control the plant induced activation of the T-DNA transfer process of Agrobacterium tumefaciens. Cell 46: 325-333.

Stachel, S. E., Nester, E. W. and Zamrisky, P. (1986). A plant cell factor induces Agrobacterium tumefaciens vir gene expression. Proc. Natl. Acad. Sci. U.S.A. 83: 379-383.

Street, H. E. (1969). Growth in organised and unorganised systems-knowledge gained by culture of organs and tissue explants. In: Plant Physiology (ed. Steward, F. C.) vol. 58 pp. 3-224. Academic Press, London.

Street, H. E. (1977). The anatomy and physiology of morphogenesis. Studies involving tissue and cell cultures In: La culture des tissus des vegetaux. (ed. Gautheret, R. J.) pp. 20-23. Resultats generaux et realisations Mason, Paris.

Tempé, J. and Petit, A. (1982). Opine utilization by Agrobacterium. In: Molecular Biology of Plant Tumours (eds. Kahl, G., Schell, J.). Academic Press, New York, pp 451-459.

Tisserat, B. (1985). Embryogenesis, organogenesis and plant regeneration In : Plant Cell Culture: a Practical Approach (ed. Dixon, R. A.) pp.79-104. IRL Press, Oxford.

Thomashow, M. F., Nutter, R., Montoya, A. L., Gordon, M. P., Nester, E. W. (1980). Intergration and organisation of Ti plasmid sequences in crown gall tumours. Cell 19: 729-739.

Thorpe, T. A. (1980). Organogenesis in vitro: structural, physiological, and biochemical aspects. Int. Rev. Cytol. Suppl. 11 A: 71-111.

Trevelyan, W. E., Procter, D. P., Harrison, J. S. (1950). Detection of sugars on paper chromatograms. Nature 166: 444-445.

Trulson, A. J., Simpson, R. B., Shahin, E. A. (1986). Transformation of cucumber. Cucumis sativus L. plants with Agrobacterium rhizogenes. Theor. Appl. Genet 73: 11-15.

Van Larebeke, N., Genetello, Ch., Schell, J., Schilperoort, R. A., Hermans, A. K., Hernalsteens, J.-P., Van Montagu, M. (1975). Acquisition of tumour inducing ability of non-oncogenic Agrobacteria as a result of plasmid transfer. Nature 255: 742-743.

Van Larebeke, N., Genetello, Ch., Hernalsteens, J. P., De Picker, A., Zaenen, I., Messens, E., Van Montagu, M., Schell, J. (1977). Transfer of Ti plasmids between Agrobacterium strains by mobilisation with the conjugative plasmid RP4. Mol. Gen. Genet. 152: 119-124.

Villiers, T. A. (1972). In: Seed Biology (ed. Kozlowski, T. T.) vol. 2, pp. 220-281. Academic Press, New York and London.

Watson, J. D., Tooze, J. T., Kurtz, D. T. (1983). Recombinant DNA: A Short Course. W. H. Freeman and Company. New York.

Yeoman, M. M. and Macleod, A. J. (1977). Tissue (callus) cultures-techniques In: Plant Tissue and Cell Culture (ed. Street, H. E.) pp. 31-59. Blackwell Scientific Publications, Oxford.

Zaenen, I., Van Larebeke, N., Teuchy, H., Van Montagu, M., Schell, J. (1974). Supercoiled circular DNA in a crown gall inducing Agrobacterium strains. J. Mol. Biol. 86: 109-127.