Growth Medium and Environmental Studies of Sweet Potato Meristem Culture

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Abstract

The ability of three New Zealand local sweet potato (*Ipomoea batatas* L.) cultivars 'Toka Toka Gold', 'Beauregard', and 'Owairaka Red' to form plantlets *in vitro* was investigated. Meristematic tips (0.2–0.4 mm) of apical shoots from vines of the three cultivars, and from tubers of 'Owairaka Red' were cultured in modified Murashige and Skoog (1962) medium (MS medium) containing plant growth regulator (s).

Cultivars and organs of explants differed in response to exogenous levels of plant growth regulator(s) and in the rate of proliferation. Optimal regeneration occurred in liquid MS medium supplemented with BA 0.1 mg/l for 'Toka Toka Gold' and 'Owairaka Red' (from vines), and with BA 0.5 + IBA 0.1 mg/l for 'Beauregard'. For 'Owairaka Red' (from tubers), MS liquid medium with BA 0.3 mg/l, and MS liquid medium with GA_3 20 mg/l (plus other organic compounds) proliferated shoots and plantlets.

Continuous lighting inhibited the proliferation of plantlets in all three cultivars. Regeneration was strongly affected by the age of the shoots from which the explants were excised and the season when cultures were begun. Successful culture was obtained by culturing explants from young shoots in the Spring.

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Chapter One: Brief Introduction to Sweet Potato

1.1 Origin and economic importance

Sweet potato (*Ipomoea batatas* L.), native to tropical America, is one of the world's most economically important crop plants. According to FAO (1995), sweet potato production in 1995 was ranked 8th among crops grown for food with an estimated 122 million metric tons produced throughout the world. China accounts for 84% of the world sweet potato production. The area under sweet potato cultivation throughout the world was estimated at 9234 thousand hectare in 1995. Sweet potatoes are grown in nearly all parts of the tropical and subtropical world, and in warmer temperate regions.

Sweet potato is of particular importance as a food crop throughout subtropical and tropical regions. It is the most important carbohydrate sources for many millions of people, particular those in the developing nations. It is not only an efficient producer of calories but also rich in many nutrients, especially vitamins and minerals (Tsou and Hong, 1992). It is becoming a popular food in the modern diet because of its high dietary fibre content.

Agricultural statistics for 1993 showed that sweet potato be New Zealand's 9th largest crops in terms of production area with 905 hectares planted (Anon.1995). Sweet potato was the most important food crop cultivated by the pre-European Maori (Wood, 1983). In New Zealand, 'Owairaka Red', with a deep red skin and a deep yellow flesh, is the most important cultivar due to market preference for red cultivars and it's high yield. The other main cultivars are 'Toka Toka Gold', 'Caromex', 'Jewel', and 'Beauregard'.

1.2 Growth requirements and vegetative propagation

Growth of sweet potato is best at or above 24°C; when temperatures fall below 10 °C growth is severely retarded (Woolfe, 1992; Onwueme and Charles, 1994). The crop is damaged by frost and this restricts its cultivation in temperate regions to areas with a minimum frost-free period of 4–6 months, and with relatively high temperatures during this period. Sweet potato grows best where light intensity is relatively high, but at the same time both flowering and rooting are promoted by short day lengths. Optimum rainfall is 740–1000 mm per annum, with approximately 500 mm falling during the growing season. A soil pH of 5.5–6.6 is preferred.

1.3 The need for *in vitro* culture of sweet potato

One of the most important limitations to good sweet potato production is the presence of virus diseases which reduce yields by up to 78% (Hahn, 1979). Viruses transmitted by aphids, white flies (Schaefers and Terry, 1976, Beetham and Mason, 1992), and unidentified vectors (Beetham and Mason, 1992) severely affect this crop. Sweet potato feathery mottle virus has been found in all sweet potato growing countries (Clark and Moyer, 1988; Beetham and Mason, 1992). This virus appears to be fully latent in all genotypes of sweet potato. New Zealand varieties, such as 'Owairaka Red' and 'Gisborne Red', have also been infected by viruses (Over de Linden and Elliott, 1971).

Fungal diseases such as black rot (*Ceratoystis fimbriata*), scurf (*Monilochaetes infuscans*), and pink rot (*Sclerotinia sclerotiorum*) are the most significant diseases of sweet potato in New Zealand. These cause the tubers to shrivel and decay both during growth and in storage (Wood, 1983; Stewart, 1989). Presence of scurf significantly reduces product presentation and saleability with potential returns dropping from around $2.50 per kg to $1 or less (Tate, 1996). Each of these diseases can be carried over in tubers from one generation to another.

In tropical or subtropical regions, sweet potato is propagated commercially either from vine cuttings or storage tubers. In New Zealand, sweet potato is propagated from tubers (Wood, 1983) due to its short growing season. Unfortunately, vegetative propagation perpetuates viruses and fungal diseases in the progeny plants (Clark and Moyer, 1988). Furthermore, vine cuttings and storage roots are difficult to transport because of their fragility, tendency to spoil and large volume.

In the absence of effective therapeutic chemicals capable of eradicating diseases from infected plants, tissue culture techniques (especially meristem culture) have been employed to eliminate systemic diseases in vegetatively reproduced crops, such as sweet potato (Quak, 1977; Mellor and Stace-Smith, 1977; Beetham and Mason, 1992). Virus-free propagation material provides growers with a high-health crop, superior quality and potentially higher yield (Over de Linden and Elliott, 1971; Wang and Hu, 1980). Virus-free planting material can increase storage root fresh-weight yield of sweet potato by 250% (Beetham and Mason, 1992). Although virus-free material is quickly re-infected by viruses, over two generation yields are still significantly higher.

In addition, to provide a method for development, maintenance, and distribution of specific disease-free clones, micropropagation offers many advantages over conventional methods for clonal propagation independent of climatic conditions and with conservation of space and time. It also allows for germplasm conservation and genetic manipulations.

Meristem (or shoot-tip) culture, callus culture, and somatic embryogenesis culture of sweet potato have been successful (Henderson et al., 1984; Chee et al., 1992). Only meristem (or shoot-tip) culture are phenotypically true to type because of the absence of a distinct or prolonged callus stage, which increases the likelihood of genetic changes. Meristem culture of sweet potatoes has been studied previously (Alconero et al., 1975; Frison and Ng, 1982; Kuo et al., 1985; Marco and Walkey, 1992; Zamora and Gruezo, 1993). However, different cultivars differed in their response to the plant growth regulators. Meristem culture of New Zealand sweet

potato cultivars has not been previously reported in the literature. Although shoot-tip culture of New Zealand sweet potato cultivar 'Owairaka Red' has been studied (Elliott, 1969), the the size of the initiation explants used in the studied were too large to achive disease-free clones and germplasm conservation. And its regeneration rate was limited.

1.4 Objectives of this study

The objectives of this study were to investigate the growth medium and environmental factors for meristem culture of New Zealand sweet potato cultivars 'Toka Toka Gold', 'Beauregard', and 'Owairaka Red'.

Chapter Two: Literature Review

2.1 General introduction of micropropagation

Micropropagation may be defined as *in vitro* regeneration of plants from organs, tissues, cells, or protoplasts (Beversdorf, 1990). Micropropagation offers many advantages over conventional methods for the multiplication of large numbers of plants independent of climatic conditions and with conservation of space and time (Short, 1990). It also provides a method for development, maintenance, and distribution of specific pathogen-tested clones (Quak, 1977). Advances in cryobiology have extended the use of meristem tip culture in germplasm storage, and meristem tip culture has also have been adopted as an tool for gene transfer in higher plants in the field of molecular biology and gene manipulations (Kartha, 1985, Nehra and Kartha, 1994). Several reviews have been written on plant propagation through tissue cultures (Bhojwani and Razdan, 1983; Wang and Hu, 1983).

There are three possible routes available for *in vitro* propagule multiplication: these are enhanced release of axillary buds; production of adventitious shoots through organogenesis; and somatic embryogenesis (Bhojwani and Razdan, 1983; Hu and Wang, 1983; Short, 1990). The merit of using axillary bud proliferation from meristems, shoots tips, or bud cultures as a means of regeneration is that the incipient shoot has already been differentiated *in vivo*. Thus, to establish a complete plant, only elongation and root differentiation are required (Bhojwani and Razdan, 1983). Plants derived from meristems, shoot tips, and bud cultures are generally phenotypically homogenous, and potentially genetically stable (Bhojwani and Razdan, 1983; Hu and Wang, 1983). *In vitro* organogenesis and embryogenesis, in contrast, must undergo developmental changes, which usually involve the formation of callus with subsequent reorganisation into plantlets. This has not been easy to achieve in most plants (Bhojwani and Razdan, 1983; Hu and Wang, 1983). Thus in practice, most micropropagation is achieved through maintaining organized tissues by the multiplication of meristems and axillary buds.

2.2 Meristem culture

Meristem culture is a technique in which a dome-shaped portion of the meristematic region of the stem tip is dissected and inoculated on nutrient medium that supports plant growth. Meristem tips are the most effective explants for the production of the virus-free plants for a wide range of economically important crops (Quak, 1977; Wang and Hu, 1980; Wang and Charles, 1991).

The meristem is a dome of actively dividing cells, about 0.1 mm in diameter and 0.25 mm long (Cutter, 1971; Quak, 1977). As a rule, the meristem of both apical shoots and axillary buds are excised (Quak, 1977). However, the chances of growth are often so little that the meristems with one or two leaf primordia are also excised.

2.3 Factors affecting success in meristem culture

Successful meristem and shoot tip culture are dependent on a complex interaction among explants, culture media, growth regulations, culture conditions, and genotype (Read, 1992; Nehra and Kartha, 1994; Zimmerman, 1995).

2.3.1 Explant

2.3.1.1 Explant size

The size of the meristem tip explants is one of the most important factors governing its regenerative capacity and the probability of recovering virus-free plants (Quak, 1977; Kartha, 1986; Nehra and Kartha, 1994). The larger the size of the meristem cultures, the greater number of plants regenerated, while number of virus-free plants obtained is inversely proportional to the size of the meristem cultured. Stone (1963) found that carnation tips smaller than 0.2 mm were unlikely to root, while those larger than 0.75 mm produced plants that still contained mottle virus. The smaller the explant, the greater the difficulty in the establishment of growth and the promotion of shoots. The

small tips either callused or formed only roots. The small size of the explant may also have an adverse effect on the rooting ability of the shoots (Bhojwani and Razdan, 1983). To obtain virus-free stock, the meristematic dome is the preferred starting material (Wang and Charles, 1990). The size of meristem taken should take account of both the chances of meristem growth and effects of viral elimination. Meristem tips between 0.2 and 0.5 mm have the best chance of producing virus-free plants in most of the cases (Quak, 1977).

2.3.1.2 Physiological condition of the explant

An important determinant of regeneration of meristem explants however, is the physiological status of the source material. Meristem tips should, preferably, be taken from young, healthy, actively growing buds (Bhojwani and Razdan, 1983; Kartha, 1986). Usually younger tissues, such as apical or axillary shoot tips or tips of adventitious shoots will regenerate better than older and mature tissue of the same stem. Immature flower buds and inflorescence are frequently quite regenerative, but were not available for use in this study.

2.3.1.3 Growth season and condition of stock plant, and bud location

Like the conventional cutting methods, the success of shoot meristem culture is affected by the growth condition of the stock plant, and the season during which the explant is obtained. Stace-Smith (1985) found that for most potato varieties, meristem excised after dormancy (i.e. in spring or early summer), rooted more readily than those taken later in the year did. With storage organs in general, the best results may be expected when meristems are dissected at the end of their dormancy period when sprouting has begun (Hartmann and Kester, 1983; Wang and Clarles, 1991). The apical buds presumably have a stronger growth potential than axillary buds, providing a greater success rate in meristem culture (Wang and Clarles, 1991).

2.3.2 Culture medium

The culture medium has two major functions: to supply the basic nutritional ingredients for continued growth of the isolated explant; and to direct growth and development through hormonal control. The basic nutritional requirements of cultured plant cells are very similar to these normally utilised by whole plants except for the application of plant growth regulators. A culture medium includes inorganic salts, organic compounds, and (or) growth regulators, and (or) miscellaneous substances. The success in obtaining complete plants can be improved by the appropriate choice of the culture media. The major features of the culture medium to be considered are its nutrients (Murashige and Skoog, 1962; Gamborg et al., 1968; Lloyd and McCown, 1980; Driver and Kuniyuki, 1984), plant growth regulators (Bhojwani and Razdan, 1983; Krikorian, 1995), and physical nature (Bhojwani and Razdan, 1983).

2.3.2.1 Inorganic salts

Inorganic salts include the macroelements (nitrogen, phosphorus, potassium, calcium, and magnesium), and microelements (boron, cobalt, copper, manganese, iodine, iron, and zinc) (Hartmann and Kester, 1983; Bhojwani and Razdan, 1983; George et al., 1987, 1988).

Murashige and Skoog's (1962) medium (MS medium) has been used widely for a range of species in different types of culture, particularly herbaceous plants, and for tissue culture in general, as it contains all the essential elements for plant growth. It is classified as a high salt medium compared to many other formulations, with relatively high levels of nitrogen, potassium, and the micronutrients, boron and manganese. The suitability of MS medium for meristem-tip culture has been reported by many authors (Quak, 1977; Wang and Hu, 1980; Bhojwani and Razdan, 1983). A basal MS medium has been used for most meristem cultures of sweet potato (Alconero et al., 1975; Beetham and Mason, 1992). Marco and Walkey (1992) reported that MS medium was better than B5 mineral salt medium (Gamborg et al., 1968) for meristem culture of sweet potato.

2.3.2.2 Organic compounds

Carbohydrates

Sucrose is used at concentrations of 2% to 5% for most of the cultures (Bhojwani and Razdan, 1983; Hartmann and Kester, 1983). In some cases glucose can substitute for sucrose, but it is not usually superior.

Vitamins

Thiamine (0.1 to 0.5 mg/l) is almost always essential, while pyridoxine and nicotinic acid (0.5 mg/l), which are required for some plant tissues, are usually added. Myo-Inositol at 100 mg/l is beneficial in many cultures and is added routinely. Other materials sometimes beneficial include pantothenic acid (0.1 mg/l) and biotin (0.1 mg/l) (Bhojwani and Razdan, 1983; Hartmann and Kester, 1983).

2.3.3 Plant Growth regulators

In addition to the nutrients, it is generally necessary to add one or more plant growth regulators to the growth medium. Growth control in many species is exerted by the type of plant growth regulators, concentration, and the sequence in which they are supplied. Auxins and cytokinins control root, shoot, and callus formation (Skoog and Miller, 1957; Wang and Charles, 1991; Krikorian, 1995). Skoog and Miller (1957) demonstrated an apparent relationship between the auxin-cytokinin balance of the nutrient medium, and the pattern of differentiation of unorganized tobacco pith callus. It was observed that a higher cytokinin-to-auxin ratio promotes shoot formation and a higher auxin-to-cytokinin ratio favours root differentiation. At intermediate concentrations, the pith tissue developed callus. Since this discovery, many aspects of cellular differentiation and organogenesis in tissue and organ cultures have been reported to be controlled by an interaction between these two plant growth

regulators that are usually required to initiate growth or differentiation in tissue culture. Although the precise requirement for auxins and cytokinins may differ between species, manipulation of exogenous auxin and cytokinin levels commonly forms the basis of regeneration techniques for a large rank of species (Krikorian, 1995; Wang and Hu, 1983·).

The exogenous requirements for plant growth regulators vary with the system and the model of shoot multiplication (Murashige, 1977; Bhojwani and Rezdan, 1983), and their endogenous levels in the plant system which is variable with the tissue, plant type, and the phase of plant growth (Bhojwani and Rezdan, 1983; Wang and Charles, 1991). Sometimes cytokinin alone is enough for optimal shoot multiplication. Consequently, for shoot multiplication the presence of an auxin in the medium is not obligatory.

Gibberellins have sometimes been used to induce shoot elongation, but are not used routinely in most cultures (Bhojwani and Rezdan, 1983; Hu and Wang, 1983; Hartmenn et al., 1990; Krikorian, 1995). For meristem culture of sweet potato gibberellins have been supplied in media (Elliott, 1969; Rey and Myoginski, 1985; Zamora and Gruezo, 1993).

2.3.3.1 Auxins

Auxins are involved with elongation of stem and internodes, tropism responses, apical dominance, abscission, and rooting. In tissue cultures auxins have been used to promote cell division and root differentiation (Bhojwani and Rezdan, 1983; Wang and Charles, 1991; Krikorian, 1995). IBA, NAA, and IAA are widely used for rooting, For shoot proliferation, 2,4–D should be avoided when shoot multiplication is attempted through axillary branching or adventitious bud development (Bhojwani and Razdan, 1983), because of its strong tendency to induce callus.

2.3.3.2 Cytokinins

Cytokinins are concerned with cell division, modification of apical dominance, and shoot differentiation. In tissue culture media, cytokinins are incorporated mainly for differentiation of adventitious shoots from callus and organs (Bhojwani and Razdan, 1983; Wang and Charles, 1991; Krikorian, 1995). These compounds are also used *in vitro* for shoot proliferation by the release of axillary buds from apical dominance. Since roots are the principal site of cytokinin biosynthesis (Letham, 1994), the majority of published culture media are supplemented with a cytokinin to support axillary shoot proliferation (Wang and Charles, 1991; Beetham and Mason, 1992).

Three cytokinins are frequently used to supplement culture media: BA, kinetin, and 2ip (Bhojwani and Razdan, 1983). In some case, 2ip was more effective than either BA or kinetin (Anderson, 1975; Cohen, 1980). However, Hutchinson (1984) found that among BA, zeatin, and kinetin, BA was the most effective, while kinetin was the least effective, and that the response was dependent on cytokinin concentration and light level. Lundergan and Janick (1980) reported that there were different promotive effects of BA, kinetin and 2ip on stimulating proliferation and growth (elongation) of 'Golden delicious' shoot-tips. Bhojwani and Razdan (1983) concluded that BA is the most widely applicable and also the cheapest cytokinin. The authors suggested that BA should be tested first for a new system. Zeatin, while occurring naturally as a potent cytokinin, is not widely used because of its high cost.

2.3.4 Physical nature of medium

For solid media, agar has been used almost exclusively as the gelling agent. For plant tissue culture work, a bacteriological grade of agar, such as Difco Bacto agar, has been widely used. Two factors that affect gelling are agar concentration and pH.

Agar is generally used at a concentration of 0.6%–1.0% (Hartmann and Kester, 1983). Lower concentrations allow more nutrient uptake and medium contact, but if explants

sink into the agar, aeration is impaired. Singha (1982, 1984) reported maximum shoot proliferation and growth with ornamental *Malus* 'Almey', at agar concentrations of 0.3 %. These results may be explained by the presence of inhibitors in agar (Kohlenback and Wernicke, 1978). With higher concentrations, the medium becomes hard, does not allow the diffusion of nutrients into the tissues, and tends to depress growth. Debergh et al. (1981) showed that raising the agar concentration from 0.6% to 1% helped to remove the symptoms of vitrification but drastically reduced the rate of propagation. In some studies, a high concentration of agar has been used to harden plants and to improve their ability to survive transplantation (Kartmann and Kester, 1983).

Culture media are usually adjusted to pH 5.6–5.8. At lower pH there is significant softening of the medium as a result of agar hydrolysis during autoclaving (Cohen, 1995). Acid-requiring plants prefer a pH of 4.5 (Hartmann and Kester, 1983), although sometimes the agar concentration may need to be increased to achieve adequate gelling.

For some plants, survival in a liquid medium may be better than in a solid gel (Bhojwani and Razdan, 1983). In cultures where agar medium induces callusing of the explants the use of a liquid medium is recommended (Stone, 1963). Mellor and Stace-Smith (1969) reported that potato meristem tips were more responsive to growth regulators in liquid than in solid media. Snir and Erez (1980) found that shoots grew faster in liquid medium on an orbital shaker. These probably resulted from increased absorption of nutrients and hormones through a greater surface area. An marked advantage of using liquid shake cultures is that the shoots broke apart as they multiplied, reducing the need for manual cutting of the shoot clusters, as in solid medium. For meristem culture of sweet potato, a solid medium was generally recommended (Alconero et al., 1975; Frison and Ng, 1981; and Zamora and Gruezo, 1993), although Beetham and Mason (1992) suggested liquid medium culture.

2.3.5 Light

The photosynthesis carried out by most plant material *in vitro* is relatively low and cultures are usually dependent on an external supply of sucrose. Light is most important in plant tissue cultures for its effect on photomorphogenesis (Appelgren, 1991; Vince-Prue, 1994).

Light intensity should normally be low in the establishment stage, and should be increased during plantlet development to promote photosynthetic leaf development (Dixon, 1985). A low light intensity in the initiation phase may prevent light-stimulated tissues browning in explants with high polyphenol content. The high light intensity during incubation is advantageous for the survival rate of the cultures when shoots begin to grow and after transfer to rooting medium. The optimum light intensity for initiating cultures with shoot meristem cultures is 100 lux (Wang and Charles, 1991). The intensity can be increased to 2000 lux after 4 weeks of incubation. As the shoot reaches a length of 1 cm, the light intensity should be increased to 4000 lux. The requirement for light intensity is dependent on the type of plant (Murashige, 1974; Hartmann et al., 1990). For shoot multiplication in *Gerberas* and many other herbaceous species, optimum light intensity was 1000 lux, 300 lux being adequate for growth. Intensities at 3000 lux or more were strongly inhibitory (Murashige, 1974).

Throughout the entire incubation period, a 16-hr photoperiod is normally used to prevent dormancy (Murashige, 1977). For some species, a period of darkness is required each day for morphogenesis (Pillai and Hildebrandt, 1969, Werckmeister, 1971). However, in other cases continuous lighting had a positive effect on growth and development of shoots and roots *in vitro* (Kukulczanka et al., 1977; Jain et al., 1988)

Fluorescent lamps which produce much less far-red radiation than incandescent lamps have been the primary light source used in plant tissue culture (Kozai et al., 1992; Vince-Prue, 1994).

2.3.6 Temperature

Culture room (incubator) temperature is usually maintained at about 25°C (Dixon, 1985, Murashige, 1974). Some species may require various temperature treatments for optimum growth. Subtropical plants grow best at 29°C.

2.3.7 Gaseous atmosphere

The rate of gas exchange from a container is important (Ziv, 1990, 1995; Navarro et al., 1994). This is related to closure of the lid and the porosity of the container (Jackson et al., 1991; Cassells and Roche, 1994). *In vitro* plants, exposed to a high photosynthetic photon flux density (PPFD) and good ventilation, showed the best *ex vitro* development (Cassells and Roche, 1994). Unrestricted exchange with the atmosphere will result in contamination and desiccation of the medium. However, in poorly aerated containers, lack of oxygen and carbon dioxide exchange, and ethylene escape may cause negative effects to plants in the stage prior to acclimatisation (Cassells and Roche, 1994; Navarro et al., 1994).

2.4 General techniques of meristem or shoot-tip culture

The regeneration of plants from existing meristem-axillary and apical meristem result in the production of genetic stable plantlets (Hu and Wang, 1983; Short, 1990). In practice, a single medium is not sufficient for both *in vitro* plant multiplication and regeneration. Transferring the propagules through a series of specially designed chemical and physical environments at each developmental stage is required for success. Commercial clonal propagation of plants by culture techniques involves fulfilling four more-or-less distinct stages of production (Murashige, 1974; Debergh and Read, 1991). These include: stage 0, selection and preparation of mother plants;

stage 1, establishment of explants in culture; stage 2, multiplication of the propagules in culture; stage 3, production of rooted plants and preliminary acclimation for conditionings; stages 4, transplanting and acclimation.

Stage 0: selection and preparation of aseptic of mother plants

This stage includes choosing typical, healthy, disease-free mother plants, or virus testing/elimination (Debergh and Read, 1991). For tropical and subtropical ornamental plants, it is advisable to maintain a relatively high temperature (25°C) and a relatively low humidity (75%). In this stage, it is important that the plants do not receive water by overhead irrigation to avoid the spreading of spores. Stage 0 also includes many kinds of interventions that can made, changing the physiological status of the explants to that which is more suitable or more reliable as a starting material (Debergh and Read, 1991). The most often manipulated parameters are light, temperature, and growth regulators.

Stage 1: establishment of explant

The purpose of stage 1 is to establish axenic cultures of explants in culture medium (Hartmann and Kester, 1983; Debergh and Read, 1991; Hartmann et al., 1990). The procedures include surface sterilisation and inoculation of explants. The factors that affect the success of this stage include choice of explants, elimination of contaminants from the explants; and culture conditions, such as medium ingredients, light, temperature, and choice of explant support.

Choice of the explant is perhaps the most critical, since it must be physiologically competent to survive the initial culture and to elicit the appropriate response (Hartmann and Kester, 1983; Debergh and Read, 1991). For most micropropagation work, apical or axillary buds are usually chosen. Younger tissues will regenerate better than more mature tissue from the same stem.

Control of explant development is achieved by manipulating the auxin and cytokinin concentrations (refer to 2.3.3). Light and temperature are not usually critical for the establishment stage (Hartmann and Kester, 1983). When plant tissues are exposed to stress situations, such as mechanical injury, metabolism of phenolic compounds is stimulated. An excellent review of ways to prevent blackening of tissues and the medium is given by George and Sherrington (1984).

Stage 2: multiplication

The function of stage 2 is to increase the number of propagules for rooting to the plantlet stage, as well as maintaining plant stock materials (Hartmann and Kester, 1983; Debergh and Read, 1991). The extended explants from stage 1 are divided, and then recultured onto a new medium. Selecting the optimum hormone levels in the culture medium is essential for maximum production of uniform plantlets (Hartmann and Kester, 1983; Krikorian, 1995).

Stage 3: preparation for transfer

The function of the pretransplant stage is to prepare plantlets for transplanting and establishment outside the artificial, closed environment of the culture vessel (Conner and Thomas 1982; Hartmann et al., 1990). Preparation may involve rooting and conditioning of the plantlets to increase its potential for acclimation and survival during transplanting. Conner and Thomas (1982) concluded the objectives of stage 3 are:

* Division of shoots and their individual rooting;
* Rendering of the plantlets capable of photoautotropic growth;
* Fulfilment of any shoot dormancy requirements;
* Attempts to confer some resistance to moisture stress and microbial infection.

The major change in the pretransplant stage is the shift to conditions that favour root initiation and shoot elongation (Hartmann and Kester, 1983). Cytokinin concentration is normally reduced or completely eliminated and auxin supply is increased. Debergh

and Maene (1981) reported that pre-treatment of *Begonia tuberybrida* shoots in auxin (IBA 2 mg/l) solution prior to transfer to *in vivo*, for about 10 days successfully produced normal plantlets. Inorganic salt concentration may be reduced which is important for various plants. Addition of phloroglucinol and phloridzin has improved rooting and reduced callus. Increased agar concentration (1.0%–1.2%) has been used in the pretransplant stage to improve the survival of herbaceous plants after transplanting. The effects are associated with reduced growth rate and improved hardiness.

The major cost in plant propagation by tissue culture is manual labour. This is especially pronounced in the later stages when individual shoots are manipulated. Debergh and Read (1991) suggested maintaining cultures as clusters of shoots as long as possible to reduction of labour costs.

Stage 4: transplanting and acclimation

The success of any micropropagation system can only be effectively measured by the number of plants that are successfully transferred from tissue culture vessels to soil. The stage 4 involves the shift from the heterotrophic to an autotrophic culture and the acclimation of the *vitro* plantlets to the outside environment (Conner and Thomas, 1982).

Tissue culture-derived plants are similar in appearance to conventionally produced cuttings but their biochemistry, physiology and anatomy is abnormal, particularly with regard to plant-water relations (Ziv et al., 1983). Tissue culture plants are difficult to transplant for two main reasons: their heterotrophic mode of nutrition and their poor control of water loss (Conner and Thomas, 1982). The plantlet or propagule must undergo a period of acclimation to enable it to survive. Plants must become autotrophic, develop functional roots and shoots, and increase resistance to desiccation and pathogen attack.

Environmental control is essential following transplanting (Conner and Thomas, 1982; Donnan et al., 1978), key factors include:

- Maintenance of high relative humidity for 2–3 weeks to protect the plant from desiccation and enable it to initiate new roots and shoots;
- Maintenance of temperature in the range of 20–27°C for optimal growth of tissue-cultured plants;
- Providing a light intensities between 60–130 μ EM^{-2} sec^{-1} for the initial period following plantlet removal from tissue culture, followed by a gradual shift to higher light intensities;
- Creating a loose, aerated, well-drained rooting medium, which allows new roots to develop quickly;
- Protection from various pathogens until some resistance has developed;
- Control of growth after transplanting to overcome or prevent dormancy and resulting lack of growth.

2.5 Meristem culture of sweet potato

Meristem culture of sweet potatoes has been studied for the production of virus-free plantlets of elite varieties, as well as maintenance, rapid multiplication and international exchange (Alconero et al., 1975; Frison and Ng, 1981; Kuo et al., 1985; Marco and Walkey, 1992; Zamora and Gruezo, 1993; Beetham and Mason, 1992).

Culture media for meristem culture of sweet potato are usually MS medium supplied with a combination of cytokinin and auxin, or cytokinin alone. Alconero et al. (1975) found that meristematic tips (0.4–0.8 mm long) of axillary shoots of 10 sweet potato cultivars developed into complete plants in 20–50 days in MS medium supplemented with a combination of kinetin 0.5 mg/l and IAA 0.2 mg/l. However, high yields of plantlets of sweet potato were obtained with MS medium plus kinetin 1 mg/l and IAA 1 mg/l (Kuo et al., 1985). MS medium plus BA 0.5 mg/l and IAA 0.2 mg/l was used by Frison and Ng (1981). Marco and Walkey (1992) demonstrated that optimal

concentration of kinetin for sweet potato tip growth was between 2.5–5 mg/l for MS medium. These authors claimed that if the right concentration of kinetin is utilized, satisfactory results could be obtained without IAA. MS medium supplemented with three combinations of growth hormones: (a). BA 0.1 mg/l, (b). BA 0.5 mg/l, or (c). BA 0.5 mg + IBA 0.1 mg/l were recommended by Beetham and Mason (1992).

GA_3 combinaed with auxin and cytokinin has been reported in meristem culture of sweet potato (Rey and Myoginski, 1985; Zamora and Gruezo, 1993). Zamora and Gruezo (1993), using MS medium containing kinetin 1 mg/l and GA_3 1 mg/l, achived 89%–96% plantlets from axillary buds with 1–2 leaf primaria of sweet potato cultivars UPL-SP1, UPL-SP2, UPL-SP3, and UPL-SP5. While Rey and Myoginski (1985) produced plantlets (80%) in MS medium with Kinetin 0.1 mg + NAA 0.1 mg + GA_3 1 mg /l. However GA_3 is not used routinely for merstem culture of sweet potato.

Differences between the research groups may be due to a number of factors including differences in light regimes, cultivars, season of culturing, age of donor plant, size of the explant, or chemical purity.

Table 2.1 Growth media for regeneration of meristem culture of sweet potatoes (*Ipomoea batatas* L.)

Cultivars	Explant	Result	Medium	Growth regulators	Regeneration (%)	Reference
Centennial, Cherokee, Hopi, Jewel, Porto Rico, Chardon, Morada, Playera, Sunny Side, and P.I. 320448	Axillary buds (0.4–0.8 mm)	Plantlets	MS	kinetin: IAA = 0.5 : 0.2		Alconero et al., 1975
	Vines or tubers	Plantlets	MS	BA 0.1 mg, BA 0.5 mg, or BA 0.5 mg + IBA 0.1 mg/l,		Beetham and Mason, 1992
Owairka Red, 907, 888	Apical buds (0.4–2.0 mm)	Plantlets	MS	NAA 1 mg + GA_3 10 mg/l		Elliott, 1969
	Apical or axillary buds from tubers (0.25–0.4 mm)	Plantlets	MS	BA 0.5 mg + IAA 0.2 mg/l	10%–90%	Frison and Ng, 1981
AIS 0122-2, AIS35-2, I 57, I 423, CN 1108-13	Apical or axillary buds from vines, Apical buds from tubers (0.2–0.4 mm)	Plantlets	MS	BA 1 mg + IAA 1 mg/l	8%–40%	Kuo et al., 1985
	Tubes (0.3–0.6 mm)	Plantlets	MS	BA 4–8 mg + IAA 1.2 mg/l		Liao and Chung, 1979
White Start, PI 315343	Vines (3 mm)	Plantlets	MS	BA 1 mg/l, or kinetin 1 mg + IAA 1 mg/l		Liz and Conover, 1978
Georgia Red, unknown c.v.	Meristem tips (0.5 mm)	Plantlets	MS, B5	Kinetin 2.5–5 mg/l	Ms medium yields better	Marco and Walkey, 1992
	Apical meristem (0.4–0.6 mm)	Plantlets	MS, B5	Kinetin 0.1 mg + NAA 0.1 mg + GA_3 1 mg /l	80%	Rey and Myoginski, 1985
	Apical or axillary buds (2–3 mm)	Plantlets	MS	Kinetin 1 mg +IAA 1mg/l or NAA 1 mg/l	95%–100%	Scaramuzzi, 1986
UPL-SP1, UPL-SP2, UPL-SP3, UPL-SP5	Axillary buds with 1–2 leaf primaria from vines	Plantlets	MS	Kinetin 1 mg/l + GA_3 1 mg /l	89%–96%	Zamora and Gruezo, 1993

Chapter Three: Experiment One

3.1 Experimental objectives

To investigate effects of cytokinin (BA) in different concentrations and combination with auxin (IBA) for sweet potato cultivars: 'Toka Toka Gold', 'Beauregard', and 'Owairaka Red'.

3.2 Materials and methods

3.2.1 Culture materials

The experiment was conducted in the Plant Science Department, Massey University, and commenced on September 26 (early spring), 1996. Apical and axillary buds were cut from vines of sweet potato cultivars 'Toka Toka Gold', 'Beauregard', and 'Owairaka Red' grown in a greenhouse; these plants had not produced storage roots at the time of the experiment. 'Owairaka Red' shoot apices derived from the tubers were also used. Tubers were washed and allowed to sprout in darkness. Once the tubers had developed shoot sprouts approximately 10–15 cm in length, the apices of sprouts (1.5–2.0 cm), which were approximately 2 weeks old, were excised for culture.

3.2.2 Establishment and maintenance of explants

Excised shoot apices from vines and tubers were washed under running tap water for 20–30 minutes, and surface sterilised by dipping in 75% ethanol for 20 seconds and then immersing in 9% (v/v) commercial bleach (3.1% sodium hypochlorite), containing 0.01% Tween 20 (poly-oxyethylene sorbitan monolaurate) wetting agent for 15 minutes. This was followed by rinsing three times in sterilised distilled water. Meristem domes (0.2–0.4 mm) without leaf primordia were aseptically dissected from apical and axillary buds using a scalpel, and a pair of forceps under a stereomicroscope in a

laminar flow transfer cabinet, and immediately inoculated onto a culture medium described next.

3.2.3 Culture media and experimental treatments

Each culture medium consisted of a basal medium and the plant growth regulator(s). The basal medium contained the inorganic salts of Murashige and Skoog (1962) medium (MS medium, Appendix 1), with the following supplements per litre: 100 mg Myo-inositol, 1 mg thiamine HCl, 1 mg nicotinic acid, 1 mg pyridoxine HCl, and 30 g sucrose. These components comprised the basal medium for experiment 1.

The three plant growth regulator treatments were set as following. Each treatment consisted of about 30 explants

BA 0.1 mg/l;
BA 0.5 mg/l;
BA 0.5 mg + IBA 0.1 mg/l.

The pH of each medium was adjusted to 5.8, and approximately 6 ml of liquid medium was dispensed into a bottle. The bottles were then autoclaved at 105 Pa, at 121°C for 15 minutes.

Meristems derived from the vines of 'Toka Toka Gold', 'Beauregard', and 'Owairaka Red' were cultured individually in glass bottles (25 × 85 mm). A filter-paper bridge was set inside the bottle to support the explant above the liquid with the free ends of the paper immersed. The bottle was tightly sealed with an aluminium lid, with a black polypropylene diaphragm under the lid.

Meristems of 'Owairaka Red' derived from the tubers were incubated individually in the pyrex-plastic bottles (24 × 88 mm) also with a filter-paper bridge, but fitted with a slightly opaque pyrex-plastic screw on cap.

Explants were subcultured to fresh medium at monthly intervals. After 3 months of culture, explants showing differentiation into shoots and plantlets were transferred to MS medium without plant growth regulators, gelled with 0.8 % (w/v) Difco Bacto agar after adjusting the pH to 5.8.

3.2.4 Growth conditions

The explants were kept initially in darkness at room temperature for the first 2 days after being inoculated to reduce browning problem caused by oxidation of phenolic substances. They were then incubated at 25°C, under 24-hr fluorescent lighting with a mean light intensity of 32 μmol $m^{-2}s^{-1}$ at the culture level outside the bottles.

3.2.5 Observations and data records

Contaminated cultures were noted and then discarded 1–2 weeks after inoculation. The cultures were examined and data recorded for survival of explants, formation of shoots, callus and roots, and number of regeneration plantlets. Survival was assessed based on colour change of explants from pale cream to light green. Other relevant developmental responses were also examined during the culture period such as colour and abnormal appearance.

3.3 Results

3.3.1 Growth of isolated meristems of different culture media

3.3.1.1 Survival

The loss of cultures due to contamination was very low (1%) in all explants. Growth of explants was visible after 2–4 days. Surviving meristems changed from pale cream to green and grew in size. Survival in each medium was similar after 6 weeks of culturing; 82%, 87%, 88% for media containing BA 0.1 mg/l, BA 0.5 mg/l, and BA 0.5 mg + IBA 0.1 mg/l respectively (Table 3.1). The media containing BA 0.5 mg/l with or without IBA 0.1 mg/l gave the highest survival rate.

Table 3.1. Effect of culture media on survival of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivars 'Toka Toka Gold' (from vines), 'Beauregard' (from vines), 'Owairaka Red' (from vines), and 'Owairaka Red' (from tubers), 6 weeks after culturing in liquid MS medium containing BA 0.1 mg/l, BA 0.5 mg/l, or BA 0.5 mg + IBA 0.1 mg/l, under 24-hr lighting at 25 °C

Treatment	Toka Toka Gold		Beauregard		Owairaka Red (vines)		Owairaka Red (tubers)		Mean	
(mg/l)	Cultured No.	Survival No.(%)	Cultured No.	Survival No.(%)	Cultured No.	Survival No.(%)	Cultured No.	Survival No.(%)	Cultured No.	Survival No.(%)
BA 0.1	34	28 (82)	32	20 (63)	35	31 (89)	37	34 (92)	34.5	28.3(82)
BA 0.5	32	29 (90)	43	33 (77)	28	24 (86)	34	33 (97)	34.3	29.7(87)
BA 0.5 IBA 0.1	39	26 (67)	29	27 (93)	40	38 (95)	41	40 (98)	37.3	32.8(88)
Mean	35	27.6 (80)	34.7	26.7 (77)	34.3	31 (90)	37.3	35.7 (96)		

3.3.1.2 Shoot formation

BA 0.1 mg/l enhanced shoot formation for all cultivars. 'Toko Toko Gold' and 'Owairaka Red' (from vines) produced 100% and 87% shoot formation (versus callus formation), respectively, when cultured on medium containing BA 0.1 mg/l 7 weeks after incubation. Increasing BA concentration to 0.5 mg/l, especially in combination with 0.1 mg/l IBA tended to increase callus formation for all sources of explants. For 'Owairaka Red' (from tubers), percentage of shoot formation (versus callus formation) was 85%, 58%, and 10% for media containing BA 0.1 mg/l, BA 0.5 mg/l, and BA 0.5 mg + IBA 0.1 mg/l, respectively.

3.3.1.3. Multiple shoots

Overall, explants developed 48% of all shoots in clump of multiple shoots and plantlets instead of single shoot. The number of shoots per shoot clump varied from 3 to 16, and averaged 4.5. The medium containing BA 0.5 mg + IBA 0.1 mg/l increased formation of multiple shoots except for 'Owairaka Red' (from tubers) (Table 3.2). For 'Owairaka Red' (from tubers), the medium containing BA 0.1 mg/l increased multiple shoot formation, but reduced plantlet regeneration (Table 3.2, 3.3). The medium containing BA 0.5 mg/l produced less multiple shoot, but proliferated shoot and plantlets for 'Owairaka Red' (from tubers).

Table 3.2 Effect of culture media on multiple shoot formation of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L) cultivars 'Toka Toka Gold' (from vines), 'Beauregard' (from vines), and 'Owairaka Red' (from vines) and 'Owairaka Red' (from tubers), 6 weeks after culturing in liquid MS medium containing BA 0.1 mg/l, BA 0.5 mg/l, or BA 0.5 mg + IBA 0.1 mg/l, under 24-hr lighting at 25 °C.

Cultivar	Treatment (mg/l)	Shoot +plantlet No.	Multiple shoot+plantlet No.	Multiple shoot+plantlet (%)
Toka Toka Gold	BA 0.1	24	2	8
	BA 0.5	4	0	0
	BA 0.5 IBA 0.1	1	1	100
	Mean	9.6	1	
Beauregard	BA 0.1	1	1	100
	BA 0.5	0	0	0
	BA 0.5 IBA 0.1	4	4	100
	Mean	1.7	1.7	
Owairaka Red (vines)	BA 0.1	16	5	31
	BA 0.5	9	2	22
	BA 0.5 IBA 0.1	6	2	33
	Mean	10.3	3	
Owairaka Red (tubers)	BA 0.1	9	9	100
	BA 0.5	31	20	65
	BA 0.5 IBA 0.1	14	11	85
	Mean	18	13.3	
Total		119	57	48

Table 3.3 Effect of culture media on regeneration of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivars 'Toka Toka Gold' (from vines), 'Beauregard' (from vines), 'Owairaka Red' (from vines), and 'Owairaka Red' (from tubers), 15 weeks after culturing in liquid MS medium containing BA 0.1 mg/l, BA 0.5 mg/l, or BA 0.5 mg + IBA 0.1 mg/l, under 24-hr lighting at 25 °C.

Treatment	Toka Toka Gold		Beauregard		Owairaka Red (vines)		Owairaka Red (tubers)	
(mg/l)	Cultured No.	Plantlet No.(%)	Cultured No.	Plantlet No.(%)	Cultured No.	Plantlet No.(%)	Cultured No.	Plantlet No.(%)
BA 0.1	34	9 (26)	32	0	35	8 (23)	37	4 (11)
BA 0.5	32	0	43	0	28	2 (7)	34	31 (91)
BA 0.5 IBA 0.1	39	1 (3)	29	4 (14)	40	5 (13)	41	14 (34)
Mean	35	3.3 (10)	34.7	1.3 (4)	34.3	5 (15)	37.3	19.7 (53)

3.3.1.4 Regeneration

Plantlets formed within 40–60 days of incubation (Plate 3.1). After 6 weeks of culturing, a large number of established shoots grew slowly, turned brown and died within 15 weeks on the three tested culture media. Although survival rates of explants cultured in the media containing BA 0.1 mg/l, BA 0.5 mg/l, and BA 0.5 mg + IBA 0.1 mg/l were 82%, 87%, and 88% 6 weeks after culturing, shoot regeneration rates for explants in the same three media were 15%, 23%, and 16%, respectively, 15 weeks after culturing.

Callus formed on explants of 'Owairaka Red' (from tubers) in MS medium containing BA 0.5 mg/l, or BA 0.5 mg + IBA 0.1 mg/l enlarged quickly 8 weeks after culturing, and then developed multiple shoot plantlets (Plate 3.2), showing in both their high rates of callus formation (42% or 90%) and high plantlet regeneration (Table 3.3). Explants treated with BA 0.5 mg/l almost failed to regenerate except for 'Owairaka Red' (from tubers) (Table 3.3).

Vitrification of the explants occurred with 26% of developing shoots, especially in those cultured in the medium containing BA 0.5 mg/l (43%) with or without IBA 0.5 mg/l, although most of them were only slightly affected.

3.3.2 Growth of isolated meristems of different sweet potato cultivars

3.3.2.1 Survival

Meristem cultures of sweet potato 'Toka Toka Gold', 'Beauregard', 'Owairaka Red'(from vines), and 'Owairaka Red' (from tubers) showed survival frequencies of 80%, 77%, 90%, and 96%, respectively, 6 weeks after culturing (Table 3.1).

Plate 3.1 Effect of incubation period on regeneration of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivar 'Owairaka Red' (from vines), cultured in liquid MS medium containing BA 0.1 mg/l, and then transferred to the solid MS medium 12 weeks after culturing, under 24-hr lighting at 25 °C, (1) plantlet 4 weeks after culturing; (2) plantlet 9 weeks after culturing; (3) plantlet 12 weeks after culturing; (4) plantlet 15 weeks after culturing.

Plate 3.2 Effect of incubation period on formation of multiple shoots and plantlets of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivar 'Owairaka Red' (from vines), in liquid MS medium with BA 0.5 mg + IBA 0.1 mg/l under 24-hr lighting at 25 °C .

Plate 3.3 Development of ageotropically negative aerial roots, and geotropical shoots from meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivar 'Owairaka Red' (from vines), cultured in liquid MS medium containing BA 0.1 mg/l, and then transferred to solid MS medium 12 weeks after culturing under 24-hr lighting at 25 °C.

3.3.2.2 Shoot formation

Percentage of shoot formation (versus callus formation) was 57%, 24%, 34%, and 49% for 'Toka Toka Gold', 'Beauregard', 'Owairaka Red' (from vines), and 'Owairaka Red' (from tubers), respectively. In general 'Toka Toka Gold' proliferated more shoots than the other cultivars. This cultivar achieved 100% shoot formation on MS medium containing BA 0.1 mg/l.

3.3.2.3 Regeneration

There were considerable differences in regeneration capacity of the cultivars (Table 3.3). The proportion of transferable plantlets were 10%, 4%, 15%, and 53% for 'Toka Toka Gold', 'Beauregard', 'Owairaka Red' (from vines), and 'Owairaka Red' (from tubers), respectively. Enhanced regeneration capacity was found in explants of 'Owairaka Red' derived from tubers. 'Beauregard' had the lowest regeneration rate, with 73 % of shoots dying within 15 weeks after the initiation stage (Table 3.1, 3.3).

The best regeneration rates for the different cultivars resulted from three different media (Table 3.3). 'Toka Toka Gold' and 'Owairaka Red' (from vines) regenerated best in the medium containing BA 0.1 mg/l (26%, and 23% respectively), 'Owairaka Red' (from tubers) performed really well in the medium supplemented with BA 0.5 mg/l (91%). However, the only medium that supported successful 'Beauregard' regeneration (14%) was that containing BA 0.5 + IBA 0.1 mg/l.

'Owairaka Red' (from tubers) regenerated a large number of shoots and plantlets (Table 3.3), which had a high rate of multiple shoots (Table 3.2). Both 'Owairaka Red' (from vines) and 'Toka Toka Gold' expressed an intermediate capacity for plantlet formation (Table 3.3). 'Beauregard' had the lowest shoot and plantlet regeneration (Table 3.3), although all of the shoots were multiple shoots (Table 3.2).

3.3.3 Root formation

Although explants cultured in MS media containing BA 0.5 mg + IBA 0.1 mg/l developed fewer shoots and plantlets, all shoots and plantlets produced roots (Table 3.4). However, with BA 0.5 mg/l 'Toka Toka Gold', and 'Beauregard' failed to develop roots, while 91% of 'Owairaka Red' (from tubers) explants produced shoots and plantlets, with 74% of them producing roots. With BA 0.1 mg/l, 'Owairaka Red' (from vines) showed a high percentage (81%) of rooting, but 'Toka Toka Gold' had an intermediate percentage (58%) of rooting.

Up to 87% of the roots developed by explants here were ageotropic (Table 3.4, Plate 3.3), while 12% of shoots developed by explants grew geotropically within the media, or horizontally on the surface of the media.

Table 3.4. Effect of culture media on root formation of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivars 'Toka Toka Gold' (TTG) (from vines), 'Beauregard Red' (BE) (from vines), and 'Owairaka Red' (OR1) (from vines), and 'Owairaka Red' (OR2) (from tubers), 15 weeks after culturing in liquid MS medium containing BA 0.1 mg/l, BA 0.5 mg/l, or BA 0.5 mg + IBA 0.1 mg/l, under 24-hr lighting at 25°C

	Treatment (mg/l)	Rooting (%)	Normal root		Upward root	
			No.	(%)	No.	(%)
TTG	BA 0.1	58	6	43	8	57
	BA 0.5	0	0	0	0	0
	BA 0.5 IBA 0.1	100	1	25	3	75
BE	BA 0.1	0	0	0	0	0
	BA 0.5	0	0	0	0	0
	BA 0.5 IBA 0.1	100	0	0	4	100
OR1	BA 0.1	81	2	15	11	85
	BA 0.5	67	1	17	5	83
	BA 0.5 IBA 0.1	100	1	0	5	100
OR2	BA 0.1	44	0	0	4	100
	BA 0.5	74	0	0	23	100
	BA 0.5 IBA 0.1	100	1	7	14	93
Total		75	12	13	77	87

3.4 Discussion

3.4.1 Regeneration of explants

The sterilisation method and techniques used in this study were successful for regeneration of meristematic plantlets from the three New Zealand sweet potato cultivars: 'Toka Toka Gold', 'Beauregard' and 'Owairaka Red'.

Meristem culture of sweet potato has been studied previously (Alconero et al., 1975; Frison and Ng, 1981; Kuo et al., 1985; Marco and Walkey, 1992; and Zamora and Gruezo, 1993), and the reported regeneration rates were variable (Table 2.1). Using shoot meristems (0.2–0.4 mm) from tubers, Kuo et al. (1985), obtained approximately 40% (range 8%–87%) plantlets, while Frison and Ng (1981) reported regeneration rates ranging 10%–90% depending on the variety cultivated, and the physiological status of the mother plant. Zamora and Gruezo (1993), culturing shoot meristems (0.25–0.4 mm) from tubers, gained a survival rate ranging 89-96%. In this experiment, the regeneration rates achieved ranged 14%–94% in three tested cultivars (Table 3.3), which were similar to previous studies.

In this study, although the culture media followed the recommendation of Australian Centre for International Agricultural Research for meristem culture of sweet potato (Beetham and Mason, 1992), there was no an enhancement of regeneration over previous reports (Alconero et al., 1975; Frison and Ng 1981; Kuo et al., 1985; Marco and Walkey, 1992; and Zamora and Gruezo, 1993). This may be due to the small size of the meristematic explants used in this experiment. The results, as reported here, indicate that propagation *in vitro* of sweet potato by meristem culture, while possible, is very variable and it is not easily attained.

Elliott (1969), culturing shoot tips (0.8–1.2 mm) of 'Owairaka Red' in MS medium with 1 mg/l of NAA and transferred to MS medium with 40 mg/l adenine and/ or 1 mg/l kinetin, was able to obtain a 16%–47% of survival rates. Using much smaller explants (0.2–0.4 mm), this study obtained regeneration rates of 23% and 91% for explants of 'Owairaka Red' derived from vines

and tubers, respectively, for the best growth regulator treatments. These results were superior to those of Elliott (1969).

3.4.2 Differences in cultivars, and sources of the explant materials

In general 'Owairaka Red' had the highest ability to regenerate *in vitro* among the three tested cultivars, while 'Beauregard' appeared to have the lowest ability to regenerate. These results agree with those from previous studies that showed regeneration rates varied with cultivar (Elliott, 1969; Alconero et al., 1975; Frison and Ng, 1981; Kuo et al., 1985; Zamora and Gruezo, 1993).

Regeneration rate also varied with source of explant material. For 'Owairaka Red', apical buds sprouting from tubers showed a higher capacity to regenerate plantlets *in vitro* than apical or axillary buds from vines, suggesting that the morphogenetic response of explants from various parts of plants *in vitro* may be markedly different. These results were in agreement with Kuo et al. (1985), who also found that sweet potato meristem of axillary buds from vines appear to develop much more slowly than those obtained from sprouting shoots of storage roots. The differences in the response of cultured shoots obtained from vines and tubers may be ascribed to the differences in the physiological status of the shoots at the time of culturing, and may be related to endogenous plant growth regulators (Kartha, 1986).

The variation in time to develop plantlets within the same source of plant material for the same treatment may have been due to the differences in size of explants (0.2–0.4 mm). The larger the explants the better the chance of survival; small explants have a low survival rate and show slow initial growth *in vitro* (Elliott, 1969; Bhojwani and Razdan, 1983; Wang and Charles, 1991). However, large explants may result in a larger number of virus-infected plants. Therefore the size of explants should be considered for both in terms of regeneration of explants and virus elimination. For cultivars which are difficult to regenerate, larger explants are recommended to be used in combination with other treatments, such as heat or chemical treatments.

3.4.3. Treatment effects

For 'Toka Toka Gold' and 'Owairaka Red' (from vines), BA 0.1 mg/l gave the best results (Table 3.3). In the BA 0.5 mg/l medium, the three cultivars derived from vines, had high survival rates, but failed to regenerate, suggesting BA 0.5 mg/l was too high for development of the shoots. However, explants of 'Owairaka Red' (from tubers) achieved 91% regeneration rate when incubated in a medium supplemented with BA 0.5 mg/l. This result, again, illustrates that cultivars, or source of explant materials have differences in physiological status, probably in endogenous plant growth regulators (Kartha, 1986), thus the requirements for exogenous plant growth regulators showed differences. It is possible that explants requiring relevantly high concentration of cytokinins for their development have a high endogenous auxin level in their meristems.

Cytokinins promote cell division, modification of apical dominance and shoot differentiation (Wang and Charles, 1991; Krikorian, 1995). In meristem culture of sweet potato, cytokinins were added to enhance axillary shoot proliferation (Kuo et al., 1985; Beetham and Mason, 1992; Zamora and Gruezo, 1993). Beetham and Mason (1992) recommended BA 0.1 mg/l, or BA 0.5 mg/l for meristem culture of sweet potato. Our results agreed in general with the earlier reports that cytokinins play an important role in shoot proliferation.

Lack of root formation on the explants cultured with BA alone is indicative of an inhibitory effect of BA on root initiation (Takayama, 1991; Wang and Charles, 1991; Krikorian, 1995). When IBA was present in the medium, 100% of shoot rooting occurred for all the cultivars, indicating a positive effect of auxin on root formation. These findings are consistent with the general findings of the effects of auxin on proliferation of rooting (Wang and Charles, 1991; Krikorian, 1995). Reducing the cytokinin levels before rooting stimulated the elongation of sassafras shoots (Wang and Hu, 1984). For meristem culture of sweet potato, medium with BA combined with IAA were reported previously (Liao and Chuang, 1979; Frison and Ng, 1981; Kuo et al., 1985).

3.4.4 Upward root growth and effect of light

Up to 88% of the roots developed by explants were ageotropic (Table 3.4, Plate 3.3). Furthermore, 12% of shoots grew ageotropically within the media, or horizontally on the surface of the media (Plate 3.3). These phenomena were also noted by Werckmeister (1971), who found that in *Cymbidium*, shoots grew ageotropically, and most roots were negatively geotropic aerial roots, when cultured on an agar medium under 24-hr continuous lighting. The growth rate of the proliferated shoots and roots was in each case poor. However, when the culture tubers were wrapped with opaque photographic paper to obscure the light, normal shoots and roots developed. The author concluded that continuous lighting inhibited the normal development of shoots and roots, and suggested darkening the culture medium around the roots (using a charcoal culture medium) while using full light intensities for photosynthesis on the shoots. In this experiment, formation of negatively geotropic aerial roots and ageotropic shoots, and inhibition of shoot development may be influenced by the 24-hr lighting regimen. Light environment in the incubator needs to be considered in the meristem culture of sweet potato.

3.4.5 Multiple shoots

Three approaches have been followed to achieve *in vitro* shoot multiplication: through callusing, adventitious bud formation, and enhanced axillary branching (Bhojwani, and Razdan, 1983; Short, 1990). Forty eight percent of the regenerated shoots and plantlets were multiple shoots in this experiment (Table 3.2). However, all these multiple shoots may not have been produced using the same process. In the media with BA 0.5 mg/l, and BA 0.5 mg + IBA 0.1 mg/l, shoot formation (versus callus formation) for 'Owairaka Red' (from tubers) was 58% and 10%, respectively, 7 weeks after culturing, but the incident regeneration was 91% and 34%, respectively, 15 weeks after culturing (Table 3.3). These results indicated that most of the multiple shoots which occurred from 'Owairaka Red' (from tubers) explants, when cultured on MS medium supplemented with BA 0.5 mg/l with or without IBA 0.1 mg/l, were probably derived through callus stage. With the exception of 'Owairaka Red' meristems derived from tubers, regenerated multiple shoots from the rest of the treatments resulted from elongation of apical and axillary buds. This result contrasts with Scaramuzzi (1986), who noted that the apical

dominance of sweet potato existed *in vitro*, but removal of apical buds did not release the growth of axillary buds. In this experiment, proliferation of multiple shoots through enhanced release of axillary is very attractive for a rapid multiplication of new cultivars and virus-free plants.

It is well known that shoot-bud differentiation is determined by cytokinin and auxin ratio; cytokinins promote bud formation, and overcome the apical dominance and enhance the branching of axillary buds (Bhojwani, and Razdan, 1983; Wang and Charles, 1991; Krikorian, 1995). In this study, the medium with BA 0.1 mg/l proliferated multiple shoots for 'Owairaka Red' (from tubers) (Table 3.2), but did not encourage shoot and plant regeneration (Table 3.3), indicating that different concentrations of BA were probably required for formation of multiple shoots and regeneration of plantlets.

Auxin may improve culture growth although auxins do not promote axillary shoot proliferation. In this study, the medium containing BA 0.5 mg + IBA 0.1 mg/l increased formation of multiple shoots except for 'Owairaka Red' (from tubers) (Table 3.2), suggesting that cytokinin in combination with auxin had a greater effect on reducing apical dominance than cytokinin alone. Our results support those of other studies (Bhojwani and Razdan, 1983; Wang, and Charles, 1991) that the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced by culturing explants in a medium containing a suitable cytokinin at an appropriate concentration, with or without an auxin.

The lengthening of the light period from 16 hr to 24 hr reduced proliferation rates of azalea (Economou and Read, 1986) and apple (Yae et al., 1987) *in vitro*. Continuous lighting inhibited the shoots and roots growth, and removed the apical dominance (Werchmeister, 1971). Werchmeister (1971) found that under 24-hr continuous light conditions, the *in vitro* development of shoots and roots of *Cymbidium* was inhibited by light, but they continued to produce new growing points. Cassells and Roche (1994) reported that the reduction of apical dominance was incidentally related to low light and low moisture vapour transmission rates. In *Rhododendron* cultivar 'Dopey', shoot production was doubled when explants were cultured at 9 W m^{-2} compared with 18 W m^{-2} (cited by Vince-Prue, 1994). In this experiment, cultures grown in continuous light for 2–3 months failed to develop mature shoots and had stunted leaves (data not presented). The culture bottles were tightly sealed by screwing on an aluminium lid,

fitted with a black polypropylene diaphragm under the lid. Condensation of water inside the bottles was observed, thus gas exchange between internal and external environments may have been low. Furthermore, the lids of culture bottles would have shaded some of the light from the top of explants. Therefore, both proliferation of the multiple shoots and inhibition of shoot development were probably caused by continuous lighting during incubation, and /or low light incubation and low lid moisture vapour transmission rates.

3.4.6 Vitrification

Physical and chemical factors resulting in vitrification have been reported previously (Debergh, 1983; Navarro et al., 1994). Factors such as high humidity, superfluous nutrients (mineral and carbohydrates), high levels of plant growth regulators, low light intensity, low agar concentration, and low exchange of gaseous atmosphere between internal and external container are the major causes found to induce shoot malformation (Debergh, 1983; Arnold and Eriksson, 1984; Bornman and Velmann 1984; Navarro et al., 1994). Furthermore, the type of agar used (Debergh, 1983) and the plastic in the culture vessel may release toxic materials, resulting in abnormal plantlets.

Vitrification of plantlets were found to occur most commonly in cultures grown on media containing BA 0.5 mg/l with or without IBA 0.5 mg/l, and seemed be related to an increase in cytokinin concentration. High cytokinin concentration may bring about morphological abnormalities (Fonnesbech, 1974; Dencso, 1987; Gaspar et al., 1987). Debergh (1983) claimed that high cytokinin concentrations increase vitrification at low agar concentrations. However, condensation of water along the inside of the culture bottles was evident in the current experiment. Free water and high humidity, and high ethylene in the culture vessels (the latter is a reflection of the closure of the container) have been proposed as another cause of vitrification (Debergh, 1987). The gas atmosphere above the cultures, which includes ethylene, ethanol, carbon dioxide, and acetaldehyde can inhibit morphogenesis if regular gaseous exchange does not occur (Read, 1992; Navarro et al., 1994). Debergh (1987) concluded that the relative humidity and the water potential were the key factors involved in abnormal morphogenesis *in vitro*. In this experiment, the major cause of the vitrification of plantlets could be due to a combination of

factors such as high cytokinin concentration in the medium, high humidity and ethylene gas within the vessel.

Slightly vitrified plantlets could become acclimatised to survive transplanting, while highly vitrified plantlets never acclimated successfully (Arnold and Eriksson, 1984; Gaspar et al., 1987; Ziv, 1991).

3.5 Conclusion

For 'Toka Toka Gold' and 'Owairaka Red' (from vines), the best medium for regeneration was MS medium supplemented with BA 0.1 mg /l. For 'Owairaka Red' (from tubers) and 'Beauregard', the best media were MS medium containing BA 0.5 mg/l, and BA 0.5 + IBA 0.1 mg/l, respectively. Study is needed to further define the cytokinin type and concentration; and identify the cause of vitrification plantlets.

Chapter Four: Experiment Two

4.1 Experimental objectives

To investigate further the effects of cytokinin type and concentration on regeneration of explants and to compare the effects of solid and liquid medium on vitrification of plantlets for meristem culture of sweet potato cultivar 'Owairaka Red'.

4.2 Materials and methods

4.2.1 Materials

The experiment commenced on December 16 (early summer), 1996 in the Plant Science Department, Massey University. Shoot apices of sweet potato cultivar 'Owairaka Red' derived from sprouted tubers were used. The tubers covered with a piece of newspaper were allowed to sprout in a tray in a greenhouse under natural temperature and light conditions. Excised sprouting shoots that initiated in September were 3 months old.

4.2.2 Establishment and maintenance of explants

The methods for establishment and maintenance of explants, preparation of the media, and inoculation of the explants were the same as in experiment 1 (refer to 3.2.2 and 3.2.3). Glass bottles (25 × 85 mm) were used as culture vessels. The bottle was tightly sealed by screwing on an aluminium lid fitted with a black polypropylene diaphragm under the lid.

4.2.3 Treatments

Since BA 0.5 mg/l combined with IBA 0.1 mg/l led to formation of callus, auxin was omitted from this medium. BA and kinetin in different concentrations for both agar and liquid media were examined. The following plant growth regulator treatments were supplemented to MS medium

with vitamins described in experiment 1 (refer to 3.2.3) for both solid and liquid media. Each treatment consisted of 40 replicates.

BA 0.1 mg/l;
BA 0.3 mg/l;
BA 0.5 mg/l;
kinetin 1 mg/l;
kinetin 3 mg/l;
kinetin 5 mg/l.

The explants were subcultured to fresh media at monthly intervals. After 10 weeks, 20 of multiple shoots from regenerating clumps in this experiment were excised and transferred to MS medium containing BA 0.1 mg/l, 1.0 % (w/v) agar, and 0.2% (w/v) charcoal.

4.2.4 Growth conditions

The growth conditions were the same as described in experiment 1 (refer to 3.2.4) with the exception of the mean light intensity used which was increased to 52 μmol $m^{-2}s^{-1}$ at the culture level outside the bottles.

4.2.5 Observations and data records

Contaminated cultures were noted and then discarded 1–2 weeks after inoculation. The cultures were examined and data recorded for survival of explants, shoot and callus formation, and number of regeneration plantlets. Survival was assessed based on colour change of explants from pale cream to light green. Other relevant developmental responses were also recorded during the culture period, such as colour and abnormal appearance.

4.3 Results

4.3.1 Survival

Observations were made 5 weeks after culturing. The highest survival rate (95%) was obtained with the explants from liquid medium containing BA 0.1 mg/l (Table 4.1). In the liquid media, increasing kinetin concentration from 1 mg/l to 5 mg/l increased the survival rate from 52% to 90%, but increasing BA concentration from 0.1 mg/l to 0.5 mg/l decreased the survival from 95% to 78% (Table 4.1). Survival was higher when explants cultured in the liquid media containing BA than these containing kinetin. However in the solid media, survival increased as BA increased from 0.1 mg/l to 0.3 mg/l or as kinetin increased from 1 mg/l to 3 mg/l. There were no differences in survival between BA 0.3 mg/l and BA 0.5 mg/l, or between kinetin 3 mg/l and kinetin 5 mg/l in the solid media.

4.3.2 Shoot formation

Callus did not develop within 5 weeks. Usually in liquid media, BA had a larger effect on multiple shoot formation than kinetin did (Table 4.2). In solid media, kinetin had a larger effect on multiple shoot formation than BA did. Both liquid and solid media containing kinetin 5 mg/l gave the same highest rate of multiple shoot formation (86%).

When comparing the same cytokinin treatment in the different physical phases of medium, shoot elongation was larger in solid media than liquid media 5 weeks after culturing. In general, the higher the cytokinin (BA, or kinetin) concentration, the longer the shoots except for solid medium containing kinetin 5 mg/l (Figure 4.1). BA 0.5 mg/l promoted bud elongation in both solid and liquid culture.

Formation of roots was not found. Most explants stopped growth 8 weeks after culturing, and then turned brown and died 3 months after culturing (data not recorded). None of the excised shoots from multiple shoot clumps were established when cultured in MS medium containing BA 0.1 mg/l, 1.0 % (w/v) agar, and 0.2% (w/v) charcoal.

Table 4.1 Effect of culture media on survival of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivar 'Owairaka Red' (from tubers), 5 weeks after culturing in MS medium containing BA 0.1 mg/l, BA 0.3 mg/l, BA 0.5 mg/l, kinetin 1 mg/l, kinetin 3 mg/l, or kinetin 5 mg/l for both liquid and solid media under 24-hr lighting at 25 °C

Treatment (mg/l)	Liquid medium		Solid medium	
	Cultured No.	Survival (%)	Cultured No.	Survival (%)
BA 0.1	40	95	40	70
BA 0.3	40	90	40	80
BA 0.5	40	77.5	40	80
Kinetin 1	40	52.5	40	85
Kinetin 3	40	75	40	92.5
Kinetin 5	40	90	40	92.5

Table 4.2 Effect of culture media on formation of single shoot and multiple shoots of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.), cultivar 'Owairaka Red' (from tubers), 5 weeks after culturing in MS medium containing BA 0.1 mg/l, BA 0.3 mg/l, BA 0.5 mg/l, kinetin 1 mg/l, kinetin 3 mg/l, or kinetin 5 mg/l for both liquid and solid media under 24-hr lighting at 25 °C

Treatment (mg/l)	Liquid medium		Solid medium	
	Single shoots (%)	Multiple shoots (%)	Single shoots (%)	Multiple shoots (%)
BA 0.1	47	53	93	7
BA 0.3	47	53	100	0
BA 0.5	29	71	84	16
Kinetin 1	100	0	26	74
Kinetin 3	70	30	78	22
Kinetin 5	14	86	14	86

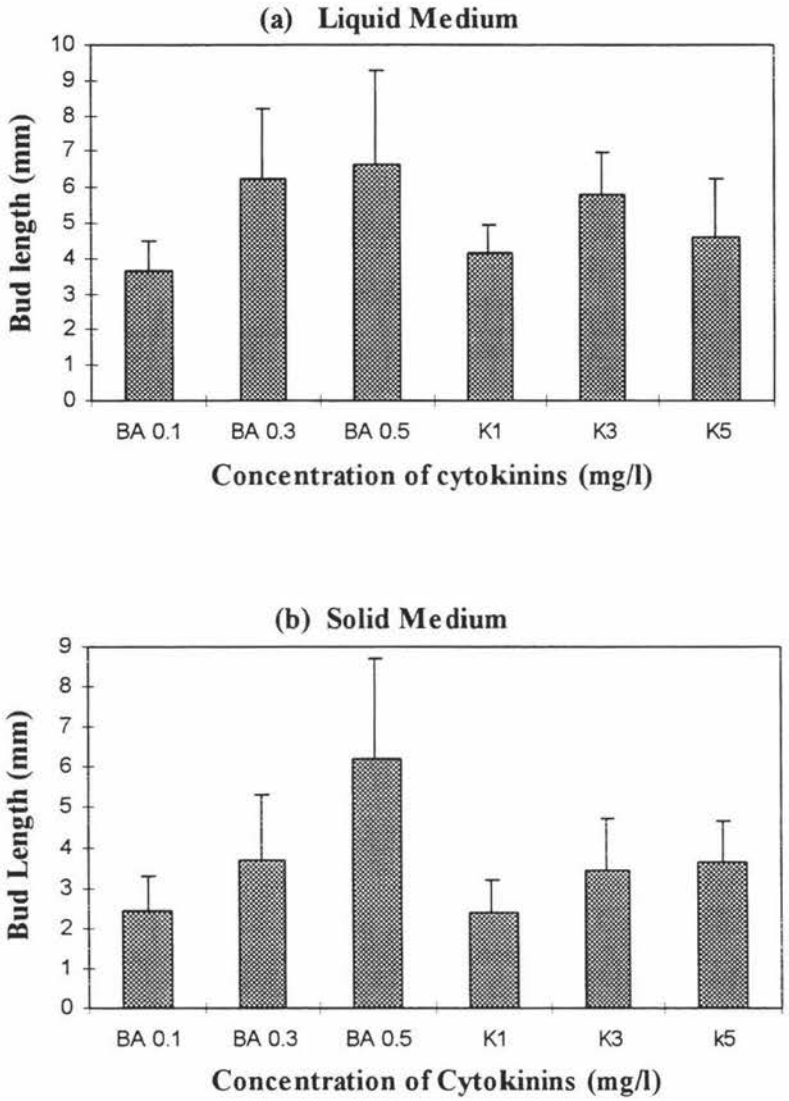

Figure 4.1 Effect of culture media on elongation of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivar 'Owairaka Red' (from tubers), 5 weeks after culturing in MS medium containing BA 0.1 mg/l (BA 0.1), BA 0.3 mg/l (BA 0.3), BA 0.5 mg/l (BA 0.5), kinetin 1 mg/l (K1), kinetin 3 mg/l (K3), or kinetin 5 mg/l (K5) for both (a) liquid and (b) solid medium, under 24-hr lighting at 25 °C

4.4 Discussion

Normally, in liquid media, BA had better effects on survival of explants and formation of multiple shoots than kinetin. Conversely, in solid media, kinetin had better effects on survival of explants and formation of multiple shoots than BA. These results suggest that BA and kinetin have different effects on survival and shoot formation. This is in partial agreement with Hempel et al. (1985), who reported that BA gave high rate of shoot proliferation in *Gerbera*, but the best shoot quality is obtained using kinetin 5–10 mg/l. Study of Hempel et al. (1985) showed that BA and kinetin had different effects on shoot proliferation and development. In this experiment, it was unexpected that both BA and kinetin would show such variable and opposing effects depending upon physical phases of the media, probably being due to differences in explants absorbing plant growth regulators between these two medium phases.

In this experiment explants cultured in liquid media supplied with BA 0.1 mg/l and BA 0.3 mg/l achieved 95% and 90% survival rates, respectively, 5 weeks after culturing. When the source of the explants are considered, these survival rates were quite similar to those seen in experiment 1 for cultures grown in media containing BA 0.1 mg/l, and BA 0.5 mg/l. However, the survival of explants from the BA 0.5 mg/l was much lower than in experiment 1 (78% versus 98%). The differences in culture conditions between experiment 1 and experiment 2 were age of the excised shoots, season of culturing, closure of culture vessels, light intensity of incubator. These issues are discussed in experiment 3 (refer to 5.4).

This study showed that both BA and kinetin supported survival of explants. Higher cytokinin concentrations increased shoot elongation (Figure 4.1), confirming the results of the previous research on cytokinins' effect on shoot growth (Wang and Charles, 1991; Krikorian, 1995). Concentrations of BA or kinetin within culture media in this study were similar to the reports by Beetham and Masom (1992) and Marco and Walkey (1992) for proliferation of shoots and plantlets for meristem culture of sweet potato. However, Liz and Conover (1978) used MS medium with much higher concentration of BA (1 mg/l) for meristem culture of sweet potato cultivar 'White Star'.

In this experiment, when the media contained lower concentrations of BA than kinetin (0.1–0.5 mg/l BA versus 1–5 mg/l kinetin), indicating that BA had a much stronger effect on promoting shoot growth than kinetin. Hutchinson (1984) reported that BA was most effective among BA, zeatin, and kinetin for organ cultures of apple. Similar results were obtained in meristem culture of *Pharbitis nil* (Bapat and Rao, 1977), and *Lilium* (Takayama, 1991). However, Scaramuzzi (1986) described shoot production efficiency of media containing IAA + kinetin, and NAA + kinetin for sweet potato, and concluded that NAA could replace IAA, but BA could not replace kinetin, contrasting with the present study.

Survival of explants was over 90% when grown with kinetin 3 mg/l in the solid medium, and kinetin 5 mg/l in both solid and liquid media. These media may have a high potential to produce regeneration of explants. Similarly, Marco and Walkey (1992) reported that for sweet potato (Georgia Red, and an unnamed cultivar), shoots proliferated with 2.5–5 mg /l kinetin without any auxin supplementation.

Considering both the survival rate and shoot growth (assessed 6 weeks after culturing), the liquid media supplemented with BA 0.3 mg/l, and BA 0.5 mg/l probably had high potential for regeneration. Since the regeneration rates of explants were not available, some important issues which may relate to this study (i.e. factors of explants, light period) will be discussed in experiment 3 (refer to 5.4).

Chapter Five: Experiment Three

5.1 Experimental objectives

To further define BA concentration in the liquid phase; to determine the effects of vessel sealing method and light period on the regeneration of explants for meristem culture of sweet potato cultivar 'Owairaka Red'.

5.2 Materials and methods

5.2.1 Materials

The experiment was commenced on March 20 (autumn), 1997 in the Plant Science Department, Massey University. Shoot apices of sweet potato cultivar 'Owairaka Red' derived from sprouted tubers were used. The tubers covered with a piece of newspaper were allowed to sprout in a tray in a greenhouse under natural temperature and light conditions. The excised sprouting shoots were approximately 3 months old.

5.2.2 Establishment and maintenance of explants

The method for establishment and maintenance of explants, preparation of the media, and inoculation of the explants were the same as described in experiment 1 (refer to 3.2.2, 3.2.3).

5.2.3 Treatments

The results from experiment 1 and 2 showed that the liquid media containing BA 0.3 mg/l, or BA 0.5 mg/l had the highest potential to promote the proliferation and regeneration. Therefore these media were selected as the culture media in this experiment.

Glass bottles were used and modified from experiment 1 and experiment 2. Two types of sealing methods (aluminium lid with foil and polypropylene film), which mainly affected gas exchange and light within the cultured bottle, were examined.

In sealing method I, the black polypropylene diaphragms under the aluminium lids of bottles were covered with shiny foil paper to reflect light within the bottles, the closure of the bottle was intermediate instead of being tight. In sealing method II, the bottles were covered firmly with a piece of sterilised polypropylene film (8 × 8 cm) and secured with a rubber band. The treatments described above were listed as following;

(1) BA 0.3 mg/l, aluminium lid with foil sealed bottle;
(2) BA 0.3 mg/l, film sealed bottle;
(3) BA 0.5 mg/l, aluminium lid with foil sealed bottle;
(4) BA 0.3 mg/l, film sealed bottle.

Each treatment consisted of 35 replications. All explants were kept in the dark at room temperature for the first two days, and then incubated under 24-hr lighting (52 μmol $m^{-2}s^{-1}$ at the culture level outside the bottles) at 25°C for the next 2 weeks.

After 2 weeks, 20 bottles of each treatment were transferred to a separate incubator at the same temperature, but the photoperiod was reduced to 16 hr (52 μmol $m^{-2}s^{-1}$ at the culture level outside the bottles). The remaining 15 bottles were maintained under the 24-hr lighting at the same temperature. Thus, the above treatments were multiplied by two, bringing the total number of treatments to eight.

All explants were transferred to fresh media 1 month after culturing. In the meantime, a new medium (Judith Toledo, 1997, International Potato Center. personal communication) treatment was added. The medium comprised of the basal medium described in experiment 1 (refer to 3.2.3), plus the following compounds per litre: GA_3 20 mg, ascorbic acid 100 mg, L-arginine hydrochloride 143 mg, calcium nitrate 4-hydrate 100 mg, putrescine dihydrochloride 25.85 mg,

calcium pantothenate 2 mg. Thus the total number of treatments were increased to nine as listed as following;

(1) 24-hr lighting, BA 0.3 mg/l, lid with foil sealed bottle, 15 explants;
(2) 24-hr lighting, BA 0.3 mg/l, film sealed bottle, 15 explants;
(3) 24-hr lighting, BA 0.5 mg/l, lid with foil sealed bottle, 15 explants;
(4) 24-hr lighting, BA 0.5 mg/l, film sealed bottle, 15 explants;
(5) 16-hr lighting, BA 0.3 mg/l, lid with foil sealed bottle, 15 explants;
(6) 16-hr lighting, BA 0.3 mg/l, film sealed bottle, 15 explants;
(7) 16-hr lighting, BA 0.5 mg/l, lid with foil sealed bottle, 15 explants;
(8) 16-hr lighting, BA 0.5 mg/l, film sealed bottle, 15 explants;
(9) 16-hr lighting, GA_3 20 mg/l (mixture of film or lid with foil sealed bottle), 20 explants (each 5 from treatments 1, 2, 3, 4).

The explants were subcultured to fresh media at monthly intervals, and 3 months after first culturing. The plantlets were transferred to MS medium gelled with 1.0% (w/v) Difco Bacto agar

5.2.4 Observations and data records

The cultures were examined and data recorded for survival of explants, formation of shoots and callus, and number of regeneration plantlets. Survival was assessed based on colour change of explants from pale cream to light green. Other relevant developmental responses were recorded during the culture period, such as colour, abnormal appearance.

5.3 Results

5.3.1 Survival of explants

All cultures were contaminate-free. As described in experiment 1, regrowth of explants was visible about 2–4 days after culturing. Surviving explants grew in size and changed colour from pale cream to green. All the explants except for treatment 2 (BA 0.3 mg/l, film sealed, 24-hr lighting), and treatment 3 (BA 0.5 mg/l, lid sealed, 24-hr lighting) achieved 100% survival rate one month after culturing (Table 5.1). Survival rate of explants was 80% for treatment 2 (BA 0.3 mg/l, film sealed, 24-hr lighting), and 93% for treatment 3 (BA 0.5 mg/l, lid sealed, 24-hr lighting).

Two months after culturing, all of the treatments with 16-hr lighting incubation maintained a 100% survival rate (Table 5.1). However, in 24-hr lighting incubation, the survival rates decreased with time with an especially large reduction in treatment 4 (BA 0.5 mg/l, film sealed 24-hr lighting), and treatment 2 (BA 0.3 mg/l, film sealed 24-hr lighting). The surviving shoots stopped growing, and remained about 0.4–0.5 mm in length.

Table 5.1 Effect of growth media, vessel sealed method and light period on survival of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivar 'Owairaka Red' (from tubers), 5 and 9 weeks after culturing in liquid MS medium containing BA 0.3 mg/l, BA 0.5 mg/l, or GA_3 20 mg/l, using sealed methods both polypropylene film and aluminium lid with foil under 16-hr or 24-hr lighting at 25 °C

Treatment	Survival of explant (5 wks)		Survival of explant (9 wks)	
	Cultured No.	Survival No. (%)	Cultured No.	Survival No. (%)
1) BA 0.3, lid, 24-hr L	15	15 (100)	15	12 (80)
2) BA 0.3, film, 24-hr L	15	12 (80)	15	9 (60)
3) BA 0.5, lid, 24-hr L	15	14 (93)	15	12 (80)
4) BA 0.5, film, 24-hr L	15	15 (100)	15	7 (47)
5) BA 0.3, lid, 16-hr L	20	20 (100)	16	16 (100)
6) BA 0.3, film, 16-hr L	21	21 (100)	16	16 (100)
7) BA 0.5, lid, 16-hr L	20	20 (100)	16	16 (100)
8) BA 0.5, film, 16-hr L	18	18 (100)	15	15 (100)
9) GA_3 20, lid, or film 16-hr L			16	16 (100)

5.3.2 Shoot and root formation

When explants were incubated under 16-hr lighting, approximately 50% of shoots formed multiple shoots in all treatments with BA in the medium. Treatment 9 (GA_3 20 mg/l, lid or film sealed, 16-hr lighting) had the highest proportion of multiple shoots (64%). When incubated under 24-hr lighting, all treatments produced only single shoots except for treatment 1 (BA 0.3, lid sealed, 24-hr lighting), in which 33% produced multiple shoots.

Formation of roots was not found except for treatments 1 and 9, while only one and two rooting plantlets were observed for treatments 9 and 1, respectively. Formation of callus was rare in this experiment. Callus was only found on 27% of explants in treatment 2 (BA 0.3, film sealed, 24-hr lighting), and 7% of explants in treatment 4 (BA 0.5, film sealed, 24-hr lighting).

5.3.3 Regeneration of explants

Regeneration of explants was observed 3 months after culturing. In 24-hr light incubation, the regeneration of explants was 27% for the treatments applied with film stopper, and 40% for the treatments applied with lid stopper regardless of BA concentrations in the growth media (Table 5.2). Overall regeneration of explants under 24-hr light incubation was 33%.

Under 16-hr lighting, Overall regeneration of explants was 63%. The hightest regeneration (88%) was obtained from treatment 5 (BA 0.3, lid sealed, 16-hr lighting), and 67%, and 69% for treatments 8 (BA 0.5, film sealed, 16-hr lighting) and 9 (GA_3 20, lid or film sealed, 16-hr lighting), respectively (Table 5.2). Treatment 7 (BA 0.5, lid sealed, 16-hr lighting) had the lowest regeneration rates.

Table 5.2 Effect of growth media, vessel sealed method, and light period on regeneration of meristematic explants (0.2–0.4 mm) of sweet potato (*Ipomoea batatas* L.) cultivar 'Owairaka Red' (from tubers), 5 and 9 weeks after culturing in liquid MS medium containing BA 0.3 mg/l, BA 0.5 mg/l, or GA_3 20 mg/l, using sealed methods both polypropylene film and aluminium lid with foil under 16-hr or 24-hr lighting at 25 °C

Treatment	Cultured No.	Plantlets No. (%)
1) BA 0.3, lid, 24-hr L	15	6 (40)
2) BA 0.3, film, 24-hr L	15	4 (27)
3) BA 0.5, lid, 24-hr L	15	6 (40)
4) BA 0.5, film, 24-hr L	15	4 (27)
5) BA 0.3, lid, 16-hr L	16	14 (88)
6) BA 0.3, film, 16-hr L	16	9 (56)
7) BA 0.5, lid, 16-hr L	16	6 (38)
8) BA 0.5, film, 16-hr L	15	10 (67)
9) GA_3 20, lid or film, 16-hr L	16	11 (69)

5.4 Discussion

5.4.1 Effects of explants

In this experiment, overall regeneration of explants and rooting ability were much lower than in experiment 1, although the same cultivar, type of explants, and plant growth regulator were used. The discrepancy between these two experiments may be predominately attributed to different physiological statuses of the explants, which were due to season when culture is commenced, and age of the donor plant, and (Bhojwani and Razdan, 1983; Kartha, 1986; Wang and Charles, 1991; Read, 1992; Nehra and Kartha, 1994). In this experiment, the culture commenced in the autumn and the initial explants were excised from sprouting shoots of 3 months old. In experiment 1, however, the explants were excised from young sprouting shoots of 2 weeks old, and the culture was commenced in the early spring.

Wang and Charles (1991) suggested that with storage organs in general, the best result may be expected when meristems are dissected at the end of their dormancy period when sprouting has begun. For most potato varieties, tips excised in spring and early summer root more readily than those taken later in the year (Quak, 1977; Wang and Charles, 1991). Similarly, in *Larix occidentalis* establishment and proliferation of explants roughly coincided with the natural summer and spring flushes (Chesick et al., 1990). The lower regeneration of explants in this experiment than in experiment 1 confirmed the previous studies and demonstrated that explants should be excised from young active shoots, and the culture should be commenced in spring or early summer for meristem culture of sweet potato 'Owairaka Red' (from tubers).

Rooting ability of explants was much poorer than in experiment 1. In addition to the season of the culture mentioned above, this phenomenon may also be because the shoots which explants were excised from were not young active shoots (Pierik and Steegmans, 1975). In this experiment, explants were excised from three-month sprouting shoots, which were very firm and dark-red in colour at the time of culturing. Rooting potential of *Rhododendron* stem segments decreased with the age of the shoots from which they were obtained (Pierik, 1969; Pierik and Steegmans, 1975). However, our results were in contrast with other

observations (Hackett, 1969; Kane and Alberty, 1989). Hackett (1969) reported that regeneration ability of bulb scales of *Lilium* was highest for the older (outer) scales.

In organ cultures of apples, browning of the explants was higher in summer and winter than spring (Hutchinson, 1984). Adult explants often produce more phenolic substances than juvenile ones (Lavarde, 1987). Poor regeneration of explants in this experiment was probably due to high phenolic content in explants (Yu and Meredith, 1986). When preparing explants in this experiment, it was observed that water used to soak excised shoots was coloured. This may have been due to phenol in the explants, but was not investigated further.

The browning caused by oxidation of phenolic compounds in explant tissue is a common problem in cultures of woody species, and tropical species (Hu and Wang, 1983; George, 1993). Physical treatments of stock plants, such as pruning to 'rejuvenate' or invigorate stock plants has been practised to stimulate new shoot growth that would be suitable for rooting as cuttings. This approach, along with sequential pruning and grafting has enabled researchers to produce more 'juvenile' growth conducive to better *in vitro* response (Franclet et al., 1987).

Explant factors, which might have a direct bearing on the meristem culture, are a consequence of the overall physiological state of the donor plant, in particular endogenous levels of plant growth regulators at various growth stages (Kartha, 1986). Yu and Meredith (1986) proposed that the connection between phenolic content and developmental position or vigour was probably related to endogenous ethylene levels.

In experiment 2, most of the explants died within 3 months of culturing, and none of the excised shoots from multiple shoot clumps were established in MS medium containing BA 0.1 mg/l, 1.0 % (w/v) agar, and 0.2% (w/v) charcoal. These may also due to unsuitable culture season and age of the donor plant as in experiment 3.

5.4.2 Effects of photoperiod

Plants with photoperiod responses may manifest these during *in vitro* culture (Murashige, 1974). In *Cymbidium*, 24-hr lighting markedly inhibited growth and development of the explants (Werckmeister, 1971). Continuous light was strongly inhibitory to sprout regeneration of *Anthurium andraeanum*, (Pierik et al., 1979). Similarly, Yae et al. (1987) reported that the internode length of apple *in vitro* was less in 24 hr than in 16 hr days. In this experiment, there was an enhancement of regeneration under 16-hr lighting compared to 24-hr lighting, confirming previous reports (Werckmeister, 1971; Pierik et al., 1979; Beetham and Mason 1992). The photoperiod used in reported studies of meristem shoot tip culture of sweet potato was all 16-hr lighting (Kuo et al., 1985; Beetham and Mason, 1992; Marco and Walkey, 1992).

By contrast, shoot regeneration from *Brassica* callus was greater in continuous light than in a daylength of 16 hr (Jain et al., 1988), Stimart and Ascher (1978) found that in lily, continuous light enhanced shoot and root formation, and darkness promoted bulb growth. Meristematic explants of *Capsicum annum* formed adventitious shoots much more rapidly under a daylength of 12 hr than in 16 hr days, or in continuous light (Phillips and Hubstenberger, 1985). Results from this experiment were not in agreement with these findings.

Light stimulates tissue browning for explants with high polyphenol content (Wang and Charles, 1991). The explants used both in this experiment and experiment 2 were more developed than those in experiments 1 were. Thus, shoots established poorly in experiment 2 and this experiment under 24-hr lighting. Paterson and Rost (1979) proposed that increased regeneration in the dark was the result of a delay in hormone destruction, which would result in a longer exposure of leaf pieces to hormones. Cytokinins are known to stimulate the synthesis of phenolic compounds (Bergmann 1964). In this experiment, the browning problem may have been made more severe as the growth media were supplemented with BA.

In this experiment, under 24-hr light condition, light was probably the most important factor inhibiting the growth and development of shoots. Light from top of the bottle was transmitted less when sealed with aluminium lid than when sealed with a translucent film, the

effect of continuous lighting, and oxidation of phenolic substances may have been weaker. Therefore, regeneration rates in the bottles sealed with aluminium lids were much greater than those with film sealed under continuous lighting. Since a dominant inhibition of continuous lighting, the plant growth regulator treatments showed no difference in this condition.

5.4.3 Effects of plant growth regulator

Under 16-hr photoperiod, lighting did not appear to limit development of explants, thus the effect of plant growth regulator showed up. Explants regenerated more in medium with BA 0.3 mg/l than that with BA 0.5 mg/l (Table 5.2).

One of the best plant growth regulator treatment (i.e. BA 0.3 mg/l) in this study was in the range of recommendation by Beetham and Mason (1992), who suggested that MS medium with BA 0.1 mg or 0.5 mg/l for meristem culture of sweet potato. However, Liz and Conover (1978), examining BA concentration ranging 0.5–2.0 mg/l, found that 1 mg/l BA produced the most proliferation for sweet potato 'White Star' shoot meristems from vines. Concentration of BA used in this experiment was much lower than that in study of Liz and Conover (1978).

GA_3 has been used in the medium to stimulate shoot elongation (Quak, 1977; Novak et al., 1980). Novak et al. (1980) reported that the addition of low concentrations of GA_3 (i.e. 0.03 mg/l) to media containing auxin and cytokinin, or cytokinin alone, have improved the growth of meristem tips, inhibited callus proliferation, and sometimes encouraged the tips to root more freely. In meristem cultures of sweet potato, GA_3 combined with auxin (Elliott, 1969; Rey and Mroginski, 1985), or cytokinin (Zamora and Gruezo, 1993) has been reported to be of use. In this experiment, after culturing in medium with BA (BA 0.3 or 0.5 mg/l) for one month, the explants were transferred to MS medium with GA_3 20 mg/l and other organic compounds (refer to 5.2.3). The explants cultured in the medium with GA_3 20 mg/l showed a high regeneration capacity, which was similar to those cultured in the medium with BA 0.3 mg/l medium. Further more GA_3 20 mg/l yielded highest multiple shoot

formation, suggesting that the medium with GA_3 had effect on proliferation of sweet potato shoots. This experimental result is similar to study by Zamora and Gruezo (1993), who showed a proliferation effect on regeneration of sweet potato in medium with GA_3 1 mg/l and kinetin 0.1 mg/l. But a much higher concentration of GA_3 (20 mg/l) was used in this experiment. The medium with BA 0.3 mg/l produced plantlets well in this experiment, suggesting that BA may replace GA3. GA_3 has been included in only 17% of the stage I media listed in Hu and Wang's review (1983). Evidently, sufficient quantities of this hormone are synthesised by most explants (Wang and Charles, 1991), which may explain why it is not always needed for explant growth.

When GA_3 is used to stimulate the growth of isolated meristems or shoot tips, it may prevent eventual root development (Murashige, 1961; Vine and Jones, 1969). Lack of rooting was found in this study for all the treatments, and has been attributed to the explants factors discussed previously. However, it is insufficient evidence to ascertain if GA_3 inhibits root formation of sweet potato. Quak (1977) suggested that plantlets formed on media containing GA_3 may need to be moved to a medium containing auxin but without GA_3, before they can be rooted.

5.4.4 Vessel closure

The type of closure also affects the photosynthetic photon flux density (PPFD), and spectral transmission (Dooley, 1991). It is known that light quality affects the morphogenesis of shoots/plantlets grown *in vitro* (Read, 1990; Seabrook, 1987). Photon ratios of red to far-red regions and of blue to red regions are, in general, important parameters for photomorphogensis (Kozai et al., 1992; Vince-Prue, 1994). In this experiment, explants cultured in vessels with aluminium lid proliferate greater than those of with polypropylene film under 24-hr lighting. Because aluminium lid transmitted less light than polypropylene film, thus inhibition of continuous lighting was weaker.

The type of vessel closure affects the gaseous composition (Short et al., 1987; Blazkova et al., 1989; Navarro et al., 1994; Ziv, 1995), as well as the light environment, hence hyperhydric change and growth of the tissues in culture such as shoot elongation,

proliferation, and fresh weight increase (Monette, 1986; Mackay and Kitto, 1988; McClelland and Smith, 1990; Jackson et al., 1991). Blazkova et al., (1989) reported that *in vitro* growth of shoots and in flowering pattern of *Chenopodium rubrum* was markedly different using different types of stoppers (cotton-wool, cellulose, cork, rubber, plastic and aluminium). This was due to changes in gaseous compositions of the vessels, such as decrease of CO_2 level and an increase of C_2H_4 (Blazkova et al., 1989; Navarro et al., 1994). Cassells and Roche (1994) reported that the use of differentially permeable membranes which have the advantage of providing a barrier to the entry of contaminants and which facilitate oxygen and carbon dioxide exchange and ethylene escape while controlling moisture loss. However, under 16-hr lighting, differences in regeneration of plantlets between the two sealed methods (aluminium lid and polypropylene film) were not found in this experiment, although differences in internal and external gas exchange between aluminium lid stopper and polypropylene film stopper may be exist. However aluminium lid stopper is preferred since it is more convenient than polypropylene film,

5.5 Conclusion

Success of meristem culture of sweet potato may be affected by season when culture is commenced, and the age of the shoots which explants were excised from. Explants should be excised from young active shoots, and the culture should be started in spring or early summer. Continuous lighting inhibited proliferation of plantlets. Proliferation of plantlets may be greater when vessels were sealed with aluminium lid than polypropylene film under 24-hr lighting.

Under16-hr lighting, meristem culture of sweet potato (*Ipomoea batatas* L.) cultivar 'Owairaka Red' (from tubers) regenerated best when cultured in liquid MS media containing BA 0.3 mg/l with culture vessel sealed by aluminium lid.

Chapter Six: Summary and Conclusions

Sweet potato is one of the world's most economically important crop plants. In this study, meristem culture of sweet potato of New Zealand cultivars 'Toka Toka Gold', 'Beauregard', and 'Owairaka Red' was investigated.

Regeneration varies between cultivars, and with the organ from which the explant is excised within the same cultivar. 'Owairaka Red' from tubers showed the highest capacity of regeneration. Optimum proliferation for 'Owairaka Red' meristem (from tubers) was obtained on medium composed of MS medium salts and vitamins supplemented with 0.3 mg/l BA. MS medium salts and vitamins with 20 mg/l GA_3 plus other vitamins and organic compounds also improved of the regeneration. The ages of shoots from which explants are taken, and time of the year starting the culture are important considerations. The most successful culture was obtained by culturing explants from young shoots in the spring season. Continuous lighting inhibited proliferation of plantlets, while 16-hr light period enhanced regeneration of plantlets.

The methodologies of meristem culture sweet potatoes presented in this study can have an important impact on the production of disease-free clonal plantlets, and international germplasm exchanges. These techniques are also applicable for quick propagation with respect to potential application of genetic manipulation in plant breeding programs. More research is needed to improve regeneration rates and develop a commercially feasible micropropagation method.

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Appendent 1 Formation of Murashige and Skoog (1962) medium (MS medium)

	MS medium (mg/l)
Macronutrients	
NH_4NO_3	20.6
KNO_3	18.8
$CaCl_2 \bullet 2H_2O$	3.0
$MgSO_4 \bullet 7H_2O$	1.5
KH_2PO_4	1.25
Micronutrients	
KI	5
H_3BO_3	100
$MnSO_4 \bullet 4H_2O$	100
$ZnSO_4 \bullet 7H_2O$	30
$Na_2MoO_4 \bullet 2H_2O$	1.0
$CuSO_4 \bullet 5H_2O$	0.1
$CoSO_4 \bullet 6H_2O$	0.1
Na_2EDTA	100
$FeSO_4 \bullet 7H_2O$	100