THE EFFECTS OF MECHANICALLY INDUCED STRESS ON *IN VIVO* AND *IN VITRO* ROSES

by

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ABSTRACT

Protocols for the successful micropropagation of 'Queen Elizabeth' ('Q.E.') and 'Dick Koster' ('D.K.') roses were established, yielding a seven-ten fold multiplication rate per month. The effects of mechanically induced stress (MIS) (shaking stress) were evaluated on early establishment of greenhouse-grown 'Q.E.' and 'D.K.' rose cuttings and the *ex vitro* survival and hardiness of micropropagated 'Q.E.' plantlets. Shaking 'Q.E.' rose cuttings at 200 rpm for 30 min daily for 4 weeks during the rooting stage increased root length, dry weight and the root:shoot dry weight ratio. Similar shaking of 'D.K.' rose at 200 rpm for 15 min increased shoot fresh and dry weight and root length and dry weight. Shaking improved the rooting of 'Q.E.' and 'D.K.' rose cuttings but its effects on early establishment need to be further assessed. Shaking micropropagated 'Q.E.' shoots or plantlets did not affect *in vitro* shoot growth. Prior to *ex vitro* acclimatization, plantlets shaken at 150 rpm for 15 min had reduced leaf dry weights. Those shaken at 200 rpm for 15 min had lower specific root water content but greater percent root dry matter. After acclimatization, some morphological changes were detected in plants shaken during the rooting stage. MIS was not directly implicated in improving *ex vitro* survival and hardiness of 'Q.E.' rose.

LES EFFETS DU STRESS INDUIT MECANIQUEMENT SUR DES CULTURES DE ROSIERS IN VIVO ET IN VITRO

Maîtrise Culture des tissus
Phytologie

RESUME

Les conditions de réussite pour la micropropagation des rosiers 'Queen Elizabeth' ('Q.E.') et 'Dick Koster' ('D.K.') ont été établies, résultant en un taux de multiplication sept à dix fois plus élevé. Les effets du stress induit mécaniquement (SIM) (stress d'agitation) ont été évalués sur l'établissement précoce des boutures de rosiers de serre 'Q.E.' et 'D.K.' ainsi que la survie et la rusticité ex vitro des plantules 'Q.E.' micropropagées. L'agitation des boutures de rosiers 'Q.E.' à 200 tour/min pendant 30 min par jour durant 4 semaines a augmenté la longueur, la masse sèche et le ratio des racines:pousses. Celle des rosiers 'D.K.' à 200 tour/min pendant 15 min a augmenté la masse fraîche et sèche des pousses ainsi que la longueur et la masse sèche des racines. L'agitation a amelioré l'enracinement des boutures de rosiers 'Q.E.' et 'D.K.' mais ses effets sur l'établissement précoce demande des essais supplémentaires. L'agitation des vitroplantes et des plantules n'a pas affecté le développement des pousses. Avant l'acclimatation ex vitro, les plantules agitées à 150 tour/min pour 15 min ont subi une réduction dans la masse sèche des feuilles. Celles qui furent agitées à 200 tour/min pendant 15 min ont subi d'une

part une réduction de la quantité d'eau spécifique des racines d'autre part une augmentation du pourcentage de la masse sèche des racines. Après l'acclimatation, quelques changements morphologiques ont été détectés dans les plantes agitées durant le stade d'enracinement. SIM n'etait pas directement impliqué dans l'amélioration de la survie et la rusticité ex vitro des rosiers 'Q.E'.

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A. FUNGICIDES:

	Common Name	Chemical Name
1.	Benomyl	methyl [1-[(butylamino)carboxyl]-1H benzimidazol-2yl] carbamate
2.	Ethazole	5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole
3.	Fenaminosulf	p-dimethylaminobenzene diazo sodium sulfonate
4.	Metalaxyl	N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-DL-alanine methyl ester

B. PLANT GROWTH REGULATORS:

	Common Name	Chemical Name	Abbreviation
1.	Indole-3-acetic acid	1H-indole-3-acetic acid	IAA
2.	Indole-3-butanoic acid	1H-indole-3-butanoic acid	IBA
3.	Naphtalene acetic acid	1-naphtalene acetic acid	NAA
4.	Gibberellic acid	GibberellinA3	GA_3
5.	Abscissic acid	[S-(Z,E)]-[5-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pentadienoic acid	ABA
6.	6-benzyl-amino purine	N-(phenyl methyl)-1H-purin-6-amine	BAP
7.	Daminozide	butanedioic acid mono (2,2-dimethylhydrazide)	-
8.	Chlormequat chloride	2-chloro-N,N,N-Trimethyl ethamnamonium chloride	-
9.	Ethephon	2-chloroethylphosphonic acid	

* CPCR, 1986.

CHAPTER I

INTRODUCTION

Many research teams have studied the effects of mechanically induced stress (MIS) on plants during the past two decades. MIS is ubiquitously present in the environment, and is caused by diverse elements such as impacting raindrops, wind and contact by animals and machinery. It can be effected indoors by rubbing (Jaffe, 1973; Jaffe *et al.*, 1984), bending or flexing stems (Beyl and Mitchell, 1983; Jaffe, 1976a; Mitchell *et al.*, 1975), shaking (Beyl and Mitchell, 1977a; Hammer *et al.*, 1974), stroking entire shoots (Suge, 1978) or by spraying water onto plants (Wheeler and Salisbury, 1979).

Roses (*Rosa* spp.) are commonly propagated asexually by cuttings, or by budding or grafting the desired scion onto selected rootstock species. Tissue culture propagation (micropropagation) of roses is currently of great commercial value to the rose industry. This technique increases the availability of rose plants with improved horticultural characteristics and is employed for the mass propagation of existing cultivars.

The objectives of this study were:

1. To establish appropriate protocols for the micropropagation of 'Queen Elizabeth' and 'Dick Koster' roses (Rosa spp.).

2. To characterize the effects of MIS (shaking stress) on the morphology of greenhouse-propagated (in vivo) and micropropagated (in vitro) roses at various propagation and transfer stages by examining plant size, growth rates and dry matter accumulation of various organs.

3. To examine the anatomy of mechanically-shaken and non-shaken in vitro and in vivo roses for quantitative differences in mechanical tissues such as collenchyma, sclerenchyma and cell wall thickness in roots, stems, leaves and petioles.

4. To determine whether MIS is useful in improving the early establishment of conventionally-propagated rose cuttings and the survival and hardiness of in vitro-derived rose plantlets.

CHAPTEP II

LITERATURE REVIEW

1. MECHANICALLY INDUCED STRESS:

1.1 Introduction:

Mechanically induced stress occurs as a natural consequence of environmental conditions as the aerial parts of plants are moved by wind, rain, irrigation, animals or machinery (Biddington, 1985a). In the laboratory or the greenhouse, the most common means of producing MIS are shaking (Walker, 1960; Beyl and Mitchell,1983), brushing (Jaffe, 1976a), vibrating using a current of air or spraying water onto plants. The term "thigmomorphogenesis" has been used to describe the plant response induced by physical contact (Jaffe, 1973) while "seismomorphogenesis" has been used to describe the effects of wind or shaking stress on plant growth (Mitchell et al, 1975). However, the effects on plant growth of these different types of stimuli seem to be the same. Different responses to MIS by several plant species have been reported. The most obvious effect

of mechanical perturbation appeared to be a reduction in stem (Beyl and Mitchell, 1977a) or petiole (Biddington and Dearman, 1985a; Heuchert and Mitchell, 1983) elongation which resulted in small, compact plants. There can also be an overall increase in the strength and hardiness of the plant. This has been interpreted as an adaptive response which enables the plant to resist physical stress (Heuchert et al., 1983).

1.2 The Effects of MIS on Plant Morphology, Anatomy and Physiology:

MIS has resulted in different morphological, anatomical and physiological changes among different plant species. The most obvious effect of MIS is growth retardation. Tomato (Lycopersicon esculentum Mill.) seedlings (Heuchert and Mitchell, 1983; Heuchert et al., 1983; Mitchell et al., 1975), rooted chrysanthemum (Chrysanthemum morifolium Ramat) cuttings (Hammer et al., 1974; Beyl and Mitchell, 1977a; Beyl and Mitchell, 1977b) and 'Alaska' pea (Pisum sativum L.) plants (Akers and Mitchell, 1984) had reduced stem elongation following shaking. The reduced height of 'Torch' and 'Bright Golden Anne' chrysanthemums was the result of shorter internodes on shaken plants, with all internodes uniformly shorter (Hammer et al., 1974).

Shoot height of bean (Phaseolus vulgaris L.) plants was inhibited by various forms of mechanical stimulation including rubbing (Biro et al., 1980; Jaffe, 1973; Jaffe, 1976a; Jaffe and Biro, 1980), wind exposure (Hunt and Jaffe, 1980) or moderate stroking (Suge, 1978). Pumpkin (Cucurbita melopepo), cucumber (Cucumis sativus) (Turgeon and Webb, 1971), sunflower (Helianthus annuus) (Beyl and Mitchell, 1983), lily (Lilium longiflorum) (Hiraki and Ota, 1975), bryony (Bryonia dioica), sensitive plant (Mimosa pudica) and castor bean (Ricinus communis) also had shorter stems than unrubbed plants (Jaffe, 1973).

Another effect which enhances the compact appearance of MIS-treated plants is the increase in radial growth of shoots. Shoot diameters of rubbed kidney bean plants (Biro et al., 1980; Hunt and Jaffe, 1980; Jaffe and Biro, 1980) and hypocotyl diameters of lettuce (Lactuca sativa L.) (Biddington and Dearman, 1985a) significantly increased compared with non-treated plants. However, periodic gyratory shaking retarded the lateral growth of tomato stems (Heuchert and Mitchell, 1983) and brushing reduced the hypocotyl diameters of cauliflower (Brassica oleracea) seedlings (Biddington and Dearman, 1985a).

Shoot fresh weights were reduced in chrysanthemum (Beyl and Mitchell, 1977a), 'Alaska' pea (Akers and Mitchell, 1984) and sunflower (Beyl and Mitchell, 1983) following shaking, and in pumpkin (Turgeon and Webb, 1971),

cauliflower, lettuce and celery (Apium graveolens) seedlings (Biddington and Dearman, 1985a) after brushing. MIS also reduced shoot dry weights in these species and in tomato (Heuchert et al. 1983; Mitchell et al., 1977).

A reduction in petiole length was observed in mechanically-stressed plants of cauliflower and celery (Biddington and Dearman, 1985a), tomato (Heuchert et al., 1983) and pumpkin (Turgeon and Webb, 1971). The petiole diameters increased in pumpkin (Turgeon and Webb, 1971), decreased in cauliflower (Biddington and Dearman, 1985a) and tomato (Heuchert et al., 1983) and were unchanged in lettuce (Biddington and Dearman, 1985a) seedlings after MIS treatment.

The root dry weights, root lengths and the number of branches per root system of cauliflower, lettuce and celery seedlings were reduced after rubbing. Also, the root:shoot dry weight ratio was increased significantly in lettuce but was reduced in celery after rubbing (Biddington and Dearman, 1985a).

Leaf number was reduced in mechanically-stressed tomato and pea (Mitchell et al., 1975), sensitive plant and bryony (Jaffe, 1973). Rubbed lettuce and celery seedlings had more leaves compared with control plants (Biddington and Dearman, 1985a). However, leaf number did not change in 'Alaska' pea subjected to shaking (Akers and Mitchell, 1984). Leaf fresh and dry weights were reduced in many

plant species that were mechanically-perturbed (Heuchert and Mitchell, 1983; Jaffe, 1973). Total leaf areas were reduced in mechanically-stressed cauliflower, lettuce, celery (Biddington and Dearman, 1985a), tomato (Heuchert and Mitchell, 1983), bryony (Jaffe, 1973), 'Alaska' pea (Akers and Mitchell, 1984) and sunflower (Beyl and Mitchell, 1983) but unchanged in pumpkin (Turgeon and Webb, 1971).

Collenchyma formation increased in petioles of celery plants exposed to wind (Venning, 1949). Cell wall thickening increased but collenchyma cell elongation decreased in thorn-apple (Datura stramonium L.) (Walker, 1960). Internode elongation decreased in rubbed bean plants. This was attributed to reduced epidermal and cortical cell elongation and reduced cell number in the vascular and pith tissues. Increased radial growth of bean stems was due to increased cortical cell expansion and the production of secondary xylem which resulted from increased cambial activity (Biro et al., 1980). A reduction in the vessel diameter in MIS-treated sweet gum (Liquidambar styraciflua) trunks was proportional to the reduction in vessel length. However, fiber length was reduced more than fiber diameter (Neel and Harris, 1971). Torque stress significantly reduced fiber length for either clockwise or counterclockwise torque treatments of red pine (Pinus resinosa Ait.) within the 1966 internode (Quirck et al.,

1975). The epidermal cuticle was thicker on rubbed than on control bean internodes (Jaffe and Biro, 1980). Lettuce seedlings had a greater density of stomata on brushed compared with unbrushed leaves (Biddington and Dearman, 1985b).

Mechanical stress may also affect developmental and physiological processes in some plants. For example, MIS caused reduced flower production and delayed flowering of marigold (Tagetes patula L.) (Jaffe, 1973; Mitchell et al., 1975) and increased the number of pistillate flowers of monoecious but not gynoecious cucumber plants (Takahashi and Suge, 1980). Respiration was not altered in tomato after shaking (Mitchell et al., 1977) but was increased in rubbed bean plants (Biro et al., 1980). Chlorophyll concentration increased in tomato after MIS treatment (Latimer and Mitchell, 1988) and in celery and lettuce seedlings after rubbing (Biddington and Dearman, 1985a). MIS treatment tended to promote resistance to water deficits or water loss in bean plants under severe drought conditions (Suge, 1980). Temporary reduction in transpiration rate and increased leaf stomatal resistance have been shown to occur for mechanically-stressed tomato plants (Mitchell et al., 1977). However, low drought resistance and high transpiration rates were detected in lettuce, cauliflower and celery seedlings after brushing (Biddington and Dearman, 1985b). Shaking or stem flexing of eggplant

(Solanum melongena L.) seedlings increased leaf water potential by 18-25 % compared with untreated plants (Latimer and Mitchell, 1988). MIS was involved in protecting tomato (Pressman et al., 1983) and celery (Pressman et al., 1984) against a physiological disorder responsible for breaking down pith parenchyma known as drought stress or gibberellin-induced stem pithiness. The mechanism of this increase in hardiness is still under investigation. Periodic gyratory shaking for 15 sec every 30 min for 30 days increased the resistance of potted poinsettia (Euphorbia pulcherima Klotzsch ex. Willd. 'Annette Hegg Diva') plants to mechanically induced leaf epinasty, but this resistance was lost within 24 hr of discontinuing shaking (Saltveit and Larson, 1983).

In conclusion, the different plant growth responses to mechanical perturbation seem to depend on several factors including: (1) plant species (Jaffe, 1973; Biddington and Dearmann, 1985a; Latimer et al., 1986), (2) type of stimulus applied (Beyl and Mitchell, 1977a; Latimer et al., 1986; Mitchell et al., 1975; Heuchert et al., 1983), (3) seed source (Jaffe, 1973), (4) time, duration and intensity of the stress stimulus (Beyl and Mitchell, 1977a; Heuchert and Mitchell, 1983), (5) season of the year (Akers and Mitchell, 1984; Heuchert and Mitchell, 1983) and (6) environmental conditions such as temperature (Jaffe, 1976a), photon flux density and dosage

of solar radiation (Akers and Mitchell, 1984; Latimer et al., 1986; Heuchert and Mitchell, 1983). The diversity of growth, developmental and physiological changes among different plant species suggests that response to mechanical stress is complex, possibly involving multiple response systems (Akers and Mitchell, 1984). Only uniform, reproducible response to mechanical treatment will permit continued progress in identifying physiological mechanisms as well as environmental determinants (Heuchert and Mitchell, 1983).

1.3 The Mechanisms of Plant Responses to MIS:

It is not yet clear how the MIS stimulus is perceived by the plant and then transferred into plant growth responses. The different mechanisms of plant responses to MIS reported in the literature include a change in plant electrical resistance, changes in the levels of endogenous growth regulators such as ethylene, auxins, gibberellins and abscissic acid and the presence of elicitors (Biddington, 1985b). Elicitors are relatively small particles of varied structure which are released in plant cells in response to stress (Takahashi and Jaffe, 1984).

Jaffe (1976b) reported a temporary decrease in electrical resistance of bean stem tissue following rubbing

which returned to the previous value within 5 min. This effect was attributed to increased plasmalemma permeability to electrolytes. However, plasmalemma permeability has not yet proven to be directly influenced by MIS.

Ethylene is one of the growth regulators that has been implicated in affecting plant growth and development when plants are exposed to MIS treatment. Ethylene reduces petiole and stem elongation and simultaneously increases radial growth in contrast to auxins and abscissic acid which inhibit extension growth only. Turgeon and Webb (1971) attributed the inhibition of petiole and stem elongation and increase in radial expansion in pumpkin to ethylene. The ethylene level increased in some plants after MIS treatment (Biro and Jaffe, 1984; Hiraki and Ota, 1975; Leopold et al., 1972; Robitaille and Leopold, 1974; Saltveit et al., 1979) but it decreased in others (Boyer et al., 1983; cited in Biddington, 1985a). Decreased ethylene levels in rubbed bryony internodes were attributed to a feed-back mechanism whereby ethylene was initially produced in very large amounts which subsequently retarded growth and inhibited further ethylene production. When ethephon, an ethylene-releasing compound, was applied to the lower nodes of bean (Erner and Jaffe, 1982) and apple (Malus domestica Borkh.) plant stems (Robitaille and Leopold, 1974), inhibition of stem elongation resulted that was comparable with the effect of MIS. However, there is some

fairly conclusive evidence suggesting that ethylene might not have any active physiological role in thigmomorphogenesis. When silver ions were sprayed as $AgNO_3$ on 'Alaska' pea, cotton (Gossypium hirsutum 'Stoneville 213') or orchid (Cattleya spp.), ethylene production was effectively blocked. However, $AgNO_3$ did not alter growth retardation caused by MIS treatment (Beyer, 1976). In addition, aminoethoxyvinylglycine, which also inhibits ethylene production, did not affect the reduction of elongation growth in mechanically-perturbed bean plants (Huberman and Jaffe, 1981; cited in Biddington, 1985a).

Ethylene might just be a co-factor involved in MIS growth responses. Ethylene does not move readily through plants in physiologically active amounts, hence it was suggested that MIS triggers ethylene production, which in turn, stops the basipetal movement of auxins and results in auxin accumulation in the tissues. This auxin accumulation in pea (Mitchell, 1977) and bean (Erner and Jaffe, 1982) plants could be responsible for inhibiting further growth. Increased bean stem flexibility following rubbing may be mediated by increased endogenous auxin (Jaffe et al., 1984).

Gibberellin (GA) levels were decreased in rubbed bean stems (Suge, 1978). Shoot tip extracts from thigmostressed sunflower plants contained no detectable GA-like substances, whereas those from unstressed controls

contained multiple zones of GA-like activity (Beyl and Mitchell, 1983). Gibberellin applications also nullified the effects of MIS on the growth and sex expression of rubbed gynoecious-type cucumber plants. This led to the assumption that GA depletion after MIS treatment suppressed plant growth (Takahashi and Suge, 1980). Moreover, GA inhibitors such as chlormequat chloride or daminozide retarded plant growth in the same way as MIS treatments (Biddington, 1985a).

Abscissic acid (ABA) may be implicated in MIS effects on some plants. Although foliar ABA levels were increased in bean plants after rubbing (Erner and Jaffe, 1982), they were not altered in cauliflower (Biddington and Dearman, 1985a) or eggplant (Latimer and Mitchell, 1988) after similar treatment.

When cells from bean plant stems were mechanically perturbed, elicitors were produced in extracellular solutions that promoted the synthesis of antifungal phytoalexins known as stress metabolites. When extracts of such elicitors were applied to bean plants, they produced the same effects as occurred in MIS treated plants. Therefore, elicitor-like substances that formed in response to MIS treatment may have caused the plant responses (Takahashi and Jaffe, 1984).

In conclusion, all of these findings suggest a hormonal mediation of mechanical stress responses. The

integrated action of plant growth regulators may indeed be responsible for the profound anatomical and physiological changes effected in plants in response to MIS treatment. However, the mechanisms of growth regulator-mediated MIS effects presented to date are still fragmented and contradictory.

1.4 Possible Uses of MIS in Crop Production:

The possible advantages and disadvantages of MIS treatment for crop production are not fully understood yet. It has been proven that MIS treatment has the potential to limit crop yield (Akers and Mitchell, 1984) but no work has yet been done to quantify these effects. An ideal application would be to enhance the positive effects of MIS on plant anatomy, such as the increased physical resistance, and suppress the negative effects such as the reduction of crop yield. This has been partially achieved in the greenhouse using various stimuli, such as shaking or rubbing, or in the field using plant growth regulators. Ultimately, there is the possibility of using genetic manipulation to produce plants whose endogenous plant growth substance compliment will ensure the required growth response (Biddington, 1985a).

Greenhouse-grown plants raised for transplantation

to the field tend to be more succulent and taller than comparable plants grown outdoors. It seems likely that greenhouse-grown plants will be less able to withstand the physical and physiological stresses which occur at or after transplantation than plants which have been mechanically-perturbed during the germination period prior to field planting. In fact, brushing is a widely used technique in Japan on sugar beet seedlings prior to transplantation to the field. MIS may also be useful in restricting plant growth prior to transplantation if field conditions are not suitable. It has been suggested that MIS would be useful to "condition" vegetable seedlings such as cauliflower, lettuce and celery prior to field planting (Biddington and Dearman, 1985a).

MIS can be substituted for the application of growth retardants to glasshouse-raised plants. Growth retardants such as A-Rest ^{R} (Ancymidol) or B-Nine SP ^{R} (Daminozide) are used commercially on floral crops such as chrysanthemum or poinsettia to produce shorter, more attractive potted flowering plants. The use of MIS treatment or growth retardants must be economically justifiable (Beyl and Mitchell, 1977b).

Commercially, brushing or bending is substituted by shaking on automated oscillatory shakers or entire growth benches, vibrating with currents of air or spraying with water. Brushing with "mechanical fingers" drawn

across crop canopies (Beyl and Mitchell, 1977a) or rubbing with a "thigmostimulator" which applies a force to a plant via a roller that moves up and down the stem (Jaffe, 1980) are also commercially effective means of providing MIS.

Plant growth regulators may reinforce or counteract MIS effects in the field. For example, gibberellic acid (GA_3) application to promote elongation growth in sugar cane (Saccharum spp.) (Nickel, 1976; cited in Biddington, 1985a) and water cress (Nasturtium spp.) (Thomas, 1982; cited in Biddington, 1985a) partially reduced the effects of naturally occurring MIS in the field. In contrast, chlormequat chloride applications on wheat (Triticum aestivum) crops to prevent lodging by shortening the stems simulated the growth response to MIS (Caldicot et al., 1974; cited in Biddington, 1985a). Since large variations are found in plant responses to plant growth regulators when tested in the field in contrast to evaluation under greenhouse or laboratory conditions, further research is required to study the modifying effects of naturally occurring MIS on plants exposed to growth regulators.

In conclusion, more research is needed to test ways of applying MIS treatments commercially and to evaluate more fully the possible benefits which might be gained from these treatments. The reports on plant growth inhibition following MIS underline the need for caution in

the design and interpretation of all experiments involving any mechanical manipulations either resulting from the plant growth environment or when growth measurements are taken. Such inadvertant application of MIS could presumably confound treatment effects and must be identical in control plantings (Mitchell et al., 1975).

2. PROPAGATION OF ROSES:

Rose plants can be propagated by seeds, cuttings, budding, grafting or through plant tissue culture. Propagation of roses by seeds is used only for producing new cultivars since the seeds vary in their genetic characteristics and will not grow true-to-type. Roses propagated by stem cuttings are often referred to as own-root plants. They are usually produced by taking semi-hardwood cuttings, and placing them in chambers that provide bottom heat of 20-25 ^{o}C and a high relative humidity supplied by fine intermittent mist. The use of rooting hormones (auxins) at the basal end of the cuttings usually helps root initiation. Under these conditions, cuttings usually root within a period of 4-6 weeks, depending on the variety. Commercially, this method is considered to be labor-

intensive and unless it is done on a large scale, it may not be cost-effective. Budding or grafting of the desired rose cultivar onto a selected rootstock is the most common procedure commercially. There are two types of budded plants. A started-eye plant is budded early and the stock top is broken over shortly after budding to force the inserted bud to start to grow. A dormant-eye plant is budded later and the stock top is not removed until planting in soil (Kiplinger, 1969). The most generally used rootstock for grafting is Rosa manetti as it is easily propagated, exhibits little or no dormancy during winter and is compatible with most greenhouse-grown rose cultivars. The scion wood is taken from flowering stems. Grafting is done either with a splice-graft or a bark-graft (Kiplinger, 1969).

Tissue culture propagation of roses is of great commercial value to the rose industry. This technique increases the availability of rose plants with improved horticultural characteristics many-fold over conventional propagation procedures and is employed for the mass propagation of existing cultivars. Tissue-cultured, own-rooted roses must be compared with conventional, vegetatively propagated rose plants for production capacity and quality (Dubois et al. 1988).

For the successful establishment of a tissue culture system, factors such as the type of explant, the

season in which the explant is obtained, the nutrient medium composition and the culture environment must be considered (Murashige, 1974). These factors are pertinent for roses at each stage of propagation: culture initiation, shoot multiplication, root initiation and ex vitro plantlet acclimatization.

Several studies have been made to establish the requirements for in vitro rose propagation (Bressan et al., 1982; Davies, 1980; Hasegawa, 1979; Hasegawa, 1980; Hyndman et al., 1982; Khosh-Khui and Sink, 1982a; Dubois et al., 1988). First, an aseptic culture free from obvious infection is initiated. At this stage, the characteristics of the explants must be well defined such as their size, physiological and ontogenetic age and the overall quality of the source plants must be carefully considered. It is necessary that a suitable proportion of the explants survive culture and proliferate rapidly (Murashige, 1974; Murashige, 1977). Two types of explants are used for in vitro propagation of roses. Terminal shoot tips of 0.5 to 2 cm (Hasegawa, 1979; Hasegawa, 1980; Jacobs et al., 1969; Khosh-Khui and Sink, 1982c; Skirvin and Chu, 1979; Mederos and Enriquez, 1987) or lateral shoot tips from the middle portion of the stem (Bressan et al., 1982; Davies, 1980) are cut from different species of greenhouse or field-grown roses. They are then surface-sterilized in 10 % Javex ^{R} (Bleach, 0.525 % sodium hypochlorite) for 20 min, and

rinsed two-three times in sterile water.

Once the plant material has been excised it is aseptically cultured on a medium which contains macro and micro elements, sugars, vitamins, agar and an adequate concentration of growth regulators to allow shoot initiation and multiplication. Shoot tips of 'Forever Yours' rose (Rosa hybrida L.) were initiated on Murashige and Skoog (1962) - MS basal medium containing 6-benzyl-amino purine (BAP; 2.0 mg/liter), naphtalene acetic acid (NAA; 0.1 mg/liter) and Staba vitamins. Linsmair and Skoog (1965) medium with an increased concentration of thiamine hydrochloride (Thiamine.HCl; 1.0 mg/liter), no other vitamins, and the same hormone concentrations was also used (Skirvin and Chu, 1979). Thiamine.HCl is an essential vitamin in culture media and is generally used in relatively low concentrations of 0.1-1.0 mg/liter (Murashige, 1977). A monthly three-fold multiplication rate was achieved from freshly excised terminal or lateral shoot tips of 'Improved Blaze' rose on MS basal medium with bacto-agar (8 g/liter), BAP (3.0 mg/liter), and indole-3-acetic acid (IAA; 0.3 mg/liter) (Hasegawa, 1979). BAP was considered to be the most effective cytokinin for rose shoot initiation and multiplication at concentrations of 1.0, 3.0 and 10.0 mg/liter (Hasegawa, 1980). Variation in shoot proliferation was observed between two new rose species (Rosa hybrida 'Tropicana' and 'Bridal Pink') and

two old world species (R. canina L. and R. damascena Mill.) on media containing a wide range of growth regulator concentrations (Khosh-Khui and Sink, 1982c). Shoot elongation was promoted with 1.0 mg/liter BAP and 1.0 mg/liter IAA (Hasegawa, 1980). Gibberellic acid inhibited shoot multiplication of 'Improved Blaze' rose at all concentrations (0.1, 0.3, 1.0, 3.0, 10.0, 30.0 mg/liter) (Hasegawa, 1980), but when it was incorporated at 0.1 mg/liter it did not cause any adverse effects on the shoot proliferation rate of seven Rosa hybrida cultivars (Davies, 1980). The addition to MS basal medium of NaH_2PO_4 is beneficial but not critical for some plants (Murashige, 1977). The shoot proliferation rate of 'Improved Blaze' rose was not affected by a NaH_2PO_4 concentration of less than 300 mg/liter but was inhibited by a concentration of 1000 mg/liter (Hasegawa, 1980). The pH of tissue culture media for roses was usually adjusted to 5.7-5.8 prior to the addition of agar.

There are no reports available on the optimization of environmental parameters (temperature, lighting) for rose cultures. Culture incubation room or growth chamber temperatures for tissue cultured roses were set at 20 ^{o}C (Davies, 1980) or 25 ^{o}C (Hasegawa, 1979), with a day length of 16 hr (Davies, 1980; Hasegawa, 1979; Hasegawa, 1980) or 24 hr (Davies, 1980) and a light intensity of 5.04 $\mu mol.m^{-2}.s^{-1}$ (Davies, 1980), 18.9 $\mu mol.m^{-2}.s^{-1}$ (Hasegawa,

1979) or 148 $\mu mol.m^{-2}.s^{-1}$ (Hasegawa, 1980). Plantlets of different rose cultivars were subcultured at 4-6 week intervals (Davies, 1980; Hyndman et al., 1982).

Successful tissue culture propagation must result in the reestablishment of a high frequency of the tissue culture-derived plants ex vitro. This involves rooting of the shoots, hardening of the plantlets to impart some tolerance to moisture stress, and their conversion from the mixotrophic to the autotrophic state. Also, the transfer of the plantlets out of culture should be done on a large scale, at a low cost and with a high survival rate (Connor and Thomas, 1981).

A reduction in the level of the MS basal salt concentration by one half or one quarter was found to enhance in vitro root formation from shoots of 'Improved Blaze' rose (Hasegawa, 1980). Also, shoots of 'Forever Yours' rose were readily rooted on one quarter-strength MS basal medium without growth regulators (Skirvin and Chu, 1979). The reduction in nitrogen concentration achieved by diluting the medium was apparently the predominant reason for improved rooting of 'Improved Blaze' rose shoots (Hyndman et al., 1982). Cytokinins were not necessary for root initiation and negatively affected subsequent transplantation of 'Improved Blaze' and 'Bridal Pink' roses into soil (Hasegawa, 1980; Hyndman et al., 1982; Khosh-Khui and Sink, 1982b). Root quality was better with

a mixture of indole-3-butanoic acid (IBA) and NAA than with IAA and NAA as IBA at 0.5 mg/liter and NAA at 0.1 mg/liter had an additive effect on root formation of 'Bridal Pink' rose (Khosh-Khui and Sink, 1982b). Root initiation was markedly affected by the length of time cultures were maintained on shoot multiplication medium prior to transfer to rooting medium. This effect was attributed to the accumulation of BAP in the shoot tissues of 'Improved Blaze' rose (Bressan et al., 1982). Khosh-Khui and Sink (1982b) found that shifting culture temperatures for 1 week at 5 ^{o}C followed by 1 week at 25-26 ^{o}C enhanced root development. The greatest number of roots was obtained with night time temperatures of 11 or 16 ^{o}C and the least number of roots was obtained with night time temperatures of 31 ^{o}C (Bressan et al., 1982). Bressan et al. (1982) also noted that a photon flux density (400-700 nm) of 66 $\mu mol.m^{-2}.s^{-1}$ for 12-24 hr daily was optimum for root initiation and for subsequent transplantation to soil of 'Improved Blaze' rose. Activated charcoal (Sigma ^{R}) in the medium (0.3 g/liter) promoted rooting of cultured shoots of the miniature rose 'Poker Chip' (Rosa spp.) after 2 weeks. The use of activated charcoal in the root initiation medium stimulated rooting, with maximum response from those shoots which had been on a shoot multiplication medium for 6 weeks. However, 'Poker Chip' rose was the only cultivar examined which responded positively to activated charcoal

treatment compared with 'Tropicana', 'Monteznma', '49er', 'Peace' and 'Mister Lincoln' rose cultivars (Rosa hybrida L.) which showed no response (Bressan et al., 1982). Rooting of rose shoots usually did not take longer than 2 weeks (Bressan et al., 1982; Hasegawa, 1979; Hasegawa, 1980; Khosh-Khui and Sink, 1982b).

The establishment of ex vitro 'Forever Yours' rose plantlets was 50 % in clay pots containing sterile vermiculite and covered by glass bottles (Skirvin and Chu, 1979). The establishment of ex vitro 'Improved Blaze' rose plantlets was 90-100 % in a potting mixture of 1 soil : 2 peat : 7 perlite (by volume) contained in 10 X 40 cm plastic pots enclosed in plastic bags and maintained in a growth chamber at 24-28 ^{o}C under high light intensity (126 $\mu mol.m^{-2}.s^{-1}$) (Hasegawa, 1980). Once established, ex vitro plantlets were transferred to the greenhouse.

Intra-species variation in Rosa hybrida (Davies, 1980; Hasegawa, 1979) and dwarf rose cultivars (Dubois et al., 1988) in addition to inter-species variation (Khosh-Khui and Sink, 1982c) occurred in tissue cultured rose shoot proliferation. This indicates the need to establish an individualized propagation medium for each species or cultivar. Reports on the micropropagation of most rose species and cultivars showed that they grew on MS basal medium amended with the appropriate growth regulator

concentrations but had relatively low multiplication rates. Although roses are widely micropropagated on a commercial basis, information concerning media and culture protocols that sustain a commercially acceptable level of performance (eight-ten-fold multiplication rate/month) is priviliged and there are no publications on this subject. Further experimental evidence is needed to assess the full potential of rose micropropagation.

CHAPTER III

IN VITRO PROPAGATION OF 'QUEEN ELIZABETH' AND 'DICK KOSTER' ROSES

3. DEVELOPMENT OF THE MICROPROPAGATION SYSTEM:

3.1 Introduction:

One of the major advantages of tissue culture techniques over conventional propagation methods is the substantial increase in the rate of multiplication, to potentially a million-fold per year. Propagule multiplication *in vitro* can occur in three ways: (1) asexual embryogenesis, (2) adventitious shoot formation and (3) enhancement of axillary shoot development (Murashige, 1977). The latter is currently of most importance to the rose industry.

The chemical composition of the nutrient medium plays a key role in plant micropropagation. The requirements for the successful tissue culture of tobacco were established by Murashige and Skoog (1962). Since then, MS basal medium has been extensively used for a wide range of

plant species with some variation in the concentrations of the vitamins and growth regulators or in the various chemical addenda such as adenine sulfate, anti-oxidants, amino acids, etc., depending on the species or cultivar and the goal of propagation (organogenesis, embryogenesis, etc.). The formation of shoots and roots in plant tissue cultures is regulated by the relative concentration of two categories of growth regulators, cytokinin and auxin. Shoot formation is usually promoted by a relatively high ratio of cytokinin to auxin whereas the inverse relationship promotes root initiation (Murashige, 1974). The shoot proliferation rate is also affected by factors such as explant type, environmental conditions and plant genotype.

Many researchers have tried to find efficient methods to propagate lines of commercial rose varieties (Bressan et al., 1982; Hasegawa, 1979; Hasegawa, 1980; Skirvin and Chu, 1979). However, the multiplication rates were not clearly reported. Studies on several rose varieties at Purdue University reported a five-fold multiplication rate per month commencing after seventeen-eighteen subcultures (Pers. comm., Arnold, 1988). Some of the problems involved with rose micropropagation have been characterized by Dr. Neville Arnold, Plant Physiologist at the Agriculture Canada Experimental Farm at l'Assomption (Pers. comm., Arnold, 1988). Explants from several cold tolerant rose cultivars consisting of nodal stem segments

with a dormant bud were successfully initiated and multiplied on a MS basal medium following testing of growth regulator concentrations. The production of phenols associated with rose culture that tend to form growth-inhibiting compounds was successfully retarded by incorporating ascorbic and citric acids into the medium at the rates of 50 and 75 mg/liter, respectively (Pers. comm., Arnold, 1988).

Reducing the MS basal salt concentration to one-half or one-quarter strength was useful for 'Improved Blaze' and 'Forever Yours' roses (Hasegawa, 1980; Skirvin and Chu, 1979). Rooting of the miniature rose 'Poker Chip' was promoted by incorporating activated charcoal into the medium at the rate of 0.3 g/liter (Bressan et al., 1982). In vitro rooting of some cold tolerant rose cultivars is done by adding activated charcoal at the rate of 0.2 g/liter (Pers. comm., Arnold, 1988). A rooting medium consisting of full-strength MS basal salts with 1 mg/liter IBA was considered satisfactory for thirty six dwarf rose culcivars (Dubois et al., 1988).

Two trials were carried out to determine appropriate micropropagation protocols for 'Queen Elizabeth' ('Q.E.') and 'Dick Koster' ('D.K.') roses. The objectives of these experiments were: (1) to optimize the cytokinin (BAP) : auxin (NAA) ratio in MS basal medium for in vitro explant establishment and shoot multiplication and

(2) to evaluate the medium advocated for root initiation by Dubois et al. (1988).

3.2 Materials and Methods:

'Queen Elizabeth' and 'Dick Koster' roses used in these studies were purchased as dormant, bare-rooted canes and potted plants, respectively, from Cramer's Nursery, Ile Perrot, Quebec. 'Queen Elizabeth' is one of the best known floribunda roses. It is an example of the now defunct grandiflora group derived from the crossing of Floribunda X Hybrid Tea (Beckett, 1984). This tall, disease resistant plant was raised in the United States by Lammerts in 1954. It produces double, clear pink flowers of the hybrid tea type, singly or in clusters on long stems springing from very vigorous upright growth (LeGrice, 1976). 'Queen Elizabeth' rose is used as an outdoor plant (Figure 3.1 A). 'Dick Koster' is a dwarf, vigorous polyantha rose (Haring, 1986). Deep pink flowers are produced in clusters on multibranched stems. It is used as an indoor flowering potted plant or as an outdoor bush (Figure 3.1 B). Both cultivars are commercially grown in Quebec.

'Queen Elizabeth' and 'D.K.' rose plants were maintained under greenhouse conditions in 12.5 cm plastic pots containing Pro-Mix BX ^{R} amended each month with 19N-6P_2O_5-12K_2O Osmocote ^{R} (3 g/liter mixture).

Figure 3.1 Greenhouse-grown 'Queen Elizabeth' (A) and 'Dick Koster' (B) rose plants (Rosa spp.)


A
B

During the winter supplemental sodium vapor lighting (5.04 $\mu mol.m^{-2}.s^{-1}$) was provided.

Nodal stem segments, 1.0-2.0 cm long with a dormant axillary bud, of greenhouse-grown 'Q.E.' and 'D.K.' roses were surface-sterilized in 10 % Javex ^{R} for 20 min, rinsed three times in sterile double distilled water and placed on MS basal medium (Appendix I) supplemented with anti-oxidants, growth regulators and agar. The anti-oxidant stock solution mixture consisted of ascorbic (5.0 g/liter) and citric (7.5 g/liter) acids. Ten ml of this mixture was added per one liter medium. The hormones were dispensed from stock solutions of BAP, NAA, IBA and GA_3 (10 mg/100 ml H_2O). The pH was adjusted to 5.7 before the addition of Anachemia ^{R} agar (6 g/liter). The media were dispensed into 25 X 150 mm test tubes (10 ml/test tube), capped with plastic covers, or 350 ml plastic containers (80 ml/container). After the media were dispensed, test tubes and containers were autoclaved at 121 ^{o}C, 1.055 $kg.cm^{-2}$ for 20 min. Cultures were kept in a completely randomized design in a walk-in culture room set at 25 $^{o}C \pm 1\ ^{o}C$, under cool white fluorescent lamps (40 W) adjusted to 50.4 $\mu mol.m^{-2}.s^{-1}$ at culture level with a 16 hr photoperiod.

In the first experiment, nodal stem segments of 'Q.E.' and 'D.K.' roses were aseptically placed on MS basal medium containing the hormone factorial combination

consisting of BAP (0, 0.1, 1.0, 2.5 and 5.0 mg/liter) and NAA (0, 0.005, 0.1, 0.25 and 0.5 mg/liter). There were four replicate test tubes containing one explant each for every medium. The time of bud break, the survival rate (number of healthy explants with or without opened buds/medium; necrotic buds were considered dead) and the number of shoots developed in each medium were recorded after bud break. Seven or 11 days after explantation, the buds (either opened with newly growing shoots or not yet opened) were aseptically severed from the explant and subcultured into the same media combinations (4 replicate test tubes/medium). The survival rate and the total number of shoots developed per single bud or shoot subcultured, in each medium, were recorded 4 weeks later. The shoot multiplication rate represents the mean number of shoots developed per single bud or shoot subcultured, in each medium, after 4 weeks.

In the second experiment, nodal stem segments of 'Q.E.' and 'D.K.' roses were aseptically placed on MS basal medium supplemented with BAP (1 mg/liter) and GA_3 (0.1 mg/liter). One week later, after bud break, the buds were severed from the explant and aseptically subcultured into the same medium . After 40 days, the shoots were subcultured into the same shoot multiplication medium or into the root initiation medium. The root initiation medium had the same composition but included 1 mg/liter IBA instead of

BAP and had no GA_3. There were eight containers for shoot multiplication with ten-twelve single shoots per container, and six containers for root initiation with eight-ten single shoots per container. The average number of shoots in the shoot multiplication medium (after 40 days) or roots in the root initiation medium (after 14 days) developed per single shoot subcultured and per container were recorded. The shoot multiplication rate was evaluated for each medium, 40 days later. After two weeks in the root initiation medium, the surviving plantlets were transferred to a potting mixture consisting of 1 perlite : 1 vermiculite : 1 Pro-Mix BX ^{R} (by volume) amended with 19N-$6P_2O_5$-$12K_2O$ Osmocote ^{R} (10 g/liter) and dolomitic limestone (10 g/liter) and sprayed with a fungicide mixture consisting of Benlate ^{R} (Benomyl) and Truban ^{R} (Ethazol) at the rates of 0.5 g/liter and 0.3 g/liter respectively. The flats, covered with clear plastic lids, were placed in a growth chamber set at 25 $\pm$ 1 ^{o}C, with a 16 hr photoperiod and a light intensity of 55.44 $\mu mol.m^{-2}.s^{-1}$ at plant level provided by cool white fluorescent lamps (20 W). The experiment was repeated twice.

3.3 Results and Discussion:

3.3.1 Experiment 1

The range of BAP and NAA concentrations (mg/liter) evaluated in the explant establishment and shoot multiplication media for 'Q.E.' and 'D.K.' roses and the medium number assigned for each combination is shown in Figure 3.2. Dormant buds from explants of 'Q.E.' developed after 7 days in culture in media 1-8 and after 11 days in media 11-13, 15-22 and 25. Bud break did not occur in media 9, 10, 14, 23 and 24. 'Queen Elizabeth' rose bud break was probably delayed by the high concentration of BAP in the culture medium (Media 11-13, 15-22 and 25). The inclusion of BAP in MS basal medium at the relatively low rates of 0.03-0.3 mg/liter also stimulated the development of 'Gold Glow' rose (Rosa hybrida) buds while BAP concentrations above 0.3 mg/liter delayed bud development (Bressan et al., 1982). In contrast, 'Improved Blaze' rose buds were promoted little if at all by the addition of any BAP to the medium and bud break was markedly delayed by BAP concentrations above 0.3 mg/liter (Bressan et al., 1982).

Figure 3.2 Diagram showing the range of BAP and NAA concentrations (mg/liter) evaluated in the explant establishment and shoot multiplication media for 'Q.E.' and 'D.K.' roses and the medium number assigned for each combination.

CONCENTRATION IN MEDIUM (mg/l)

NAA \ BAP	0	0.1	1.0	2.5	5.0
0	1	6	11	16	21
0.005	2	7	12	17	22
0.1	3	8	13	18	23
0.25	4	9	14	19	24
0.5	5	10	15	20	25

CONCENTRATION IN MEDIUM (mg/l)

All replicates (4/4) of 'Q.E.' rose survived in media 1-8, 17, 18 and 25. The survival rate was 3/4 in media 10, 15, 16, 21 and 22, and 2/4 in media 12, 13, 19 and 20. The explants in media 9, 10, 14, 23 and 24 did not survive, so these media were not subsequently used to test shoot multiplication.

Dormant buds from explants of 'D.K.' rose developed in all media within 7 days. The survival rate of 'D.K.' rose was 4/4 in the explant establishment media 1-8, 10-17, 19-21 and 25 and 3/4 in the other explant establishment media.

Early and uniform bud break and good survival of 'D.K.' rose in all media compared with 'Q.E.' rose explants, which appear to be more selective in their exogenous hormone requirements, could be species-related. The best explant establishment media for both 'Q.E.' and 'D.K.' roses were the ones with BAP:NAA rates of 0.1:0.25, 0.1:0.5, 1.0:0 and 1.0:0.005 mg/liter as they developed two to three shoots after bud break, within 7-11 days, compared with either no bud break or only one or two shoots in the other media.

All replicates (4/4) of 'Q.E.' rose buds or shoots survived when subcultured to fresh shoot multiplication media 1-7 and 11 while all the 'D.K.' shoots survived in media 1-4, 6, 8, 10 and 14-17. The survival rate of 'Q.E.' and 'D.K.' buds or shoots was 3/4 in the remaining media.

Shoot multiplication of 'Q.E.' rose was observed in twenty five different combinations of BAP and NAA in the shoot multiplication media (Figure 3.3). Shoots of 'Q.E.' cultured on media without BAP did not develop new lateral shoots. They yellowed, turned brown and died. These results are consistent with reports showing that BAP was an essential cytokinin for good shoot multiplication of roses (Davies, 1980; Hasegawa, 1980). The shoot multiplication of rose shoots which occurs through the enhancement of axillary bud development is regulated by apical dominance. Factors which antagonize this apical dominance such as exogenous cytokinins (BAP) or excision of the apex will enhance shoot multiplication (Bressan et al., 1982).

Media with BAP (0.1 mg/liter) and NAA (0 and 0.005 mg/liter) induced two to three new 'Q.E.' shoots which soon deteriorated due to excessive callus formation. In media 13, 14, 17-22 and 25 one or two new shoots or some callus developed; the leaves were shrunken and turned yellow.

The best shoot multiplication media for 'Q.E.' roses contained 1 mg/liter BAP and no NAA (medium 11) or 0.1 mg/liter of both BAP and NAA (medium 8). These media promoted an average of seven to eight shoots per initial bud or shoot subcultured, without callus formation at the shoot base, in 40 days (Figure 3.4).

Figure 3.3 The effects of MS basal medium supplemented with various BAP and NAA concentrations (mg/liter) on shoot multiplication of 'Q.E.' rose after 4 weeks in culture.

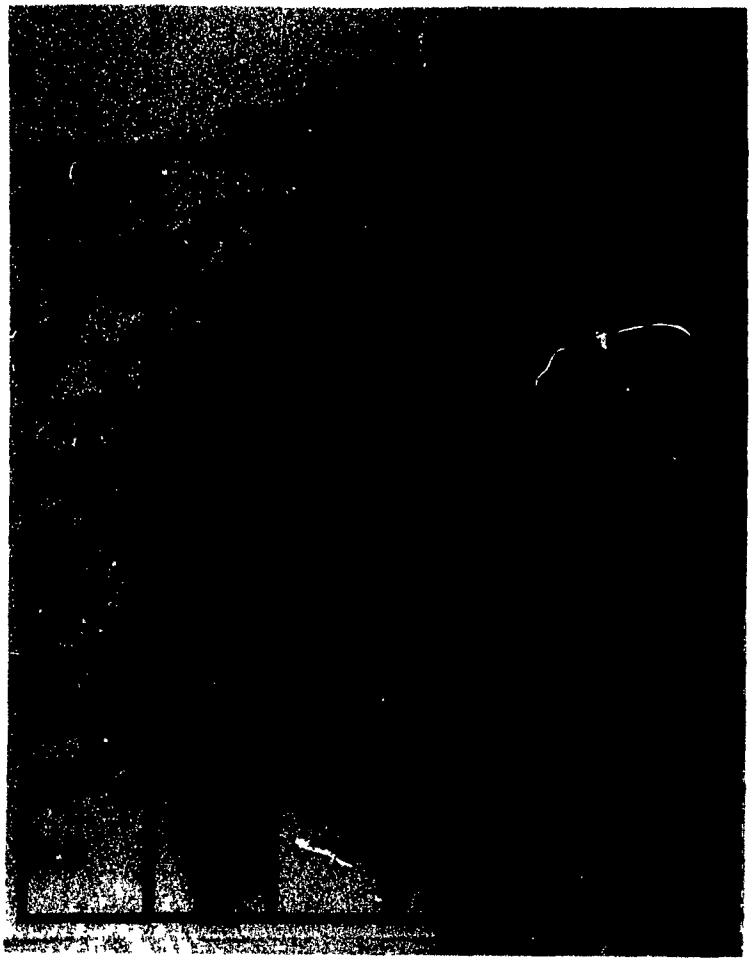
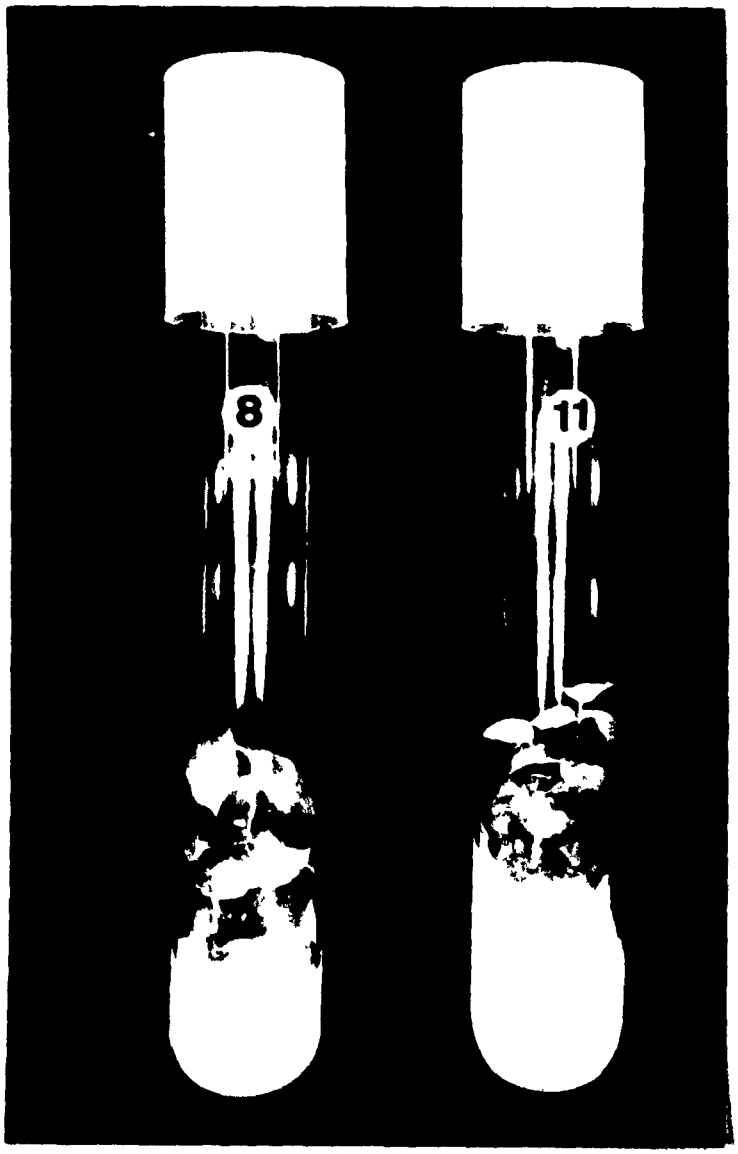


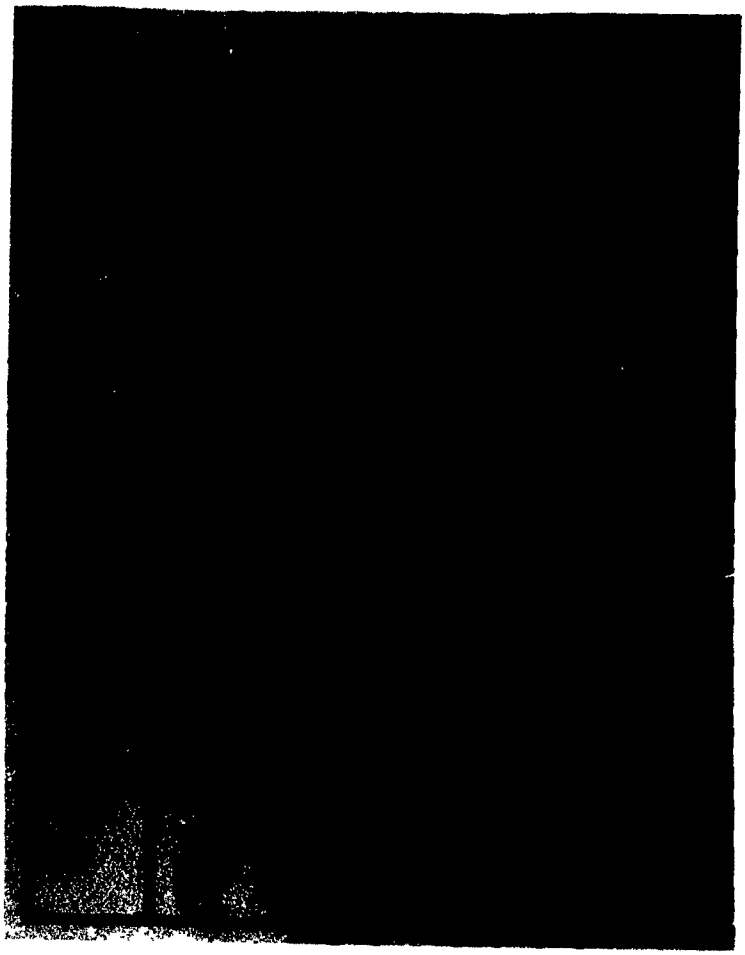
Figure 3.4 Shoot multiplication of 'Q.E.' rose on MS basal medium supplemented with 0.1 mg/liter BAP and 0.1 mg/liter NAA (medium 8) or with 1.0 mg/liter BAP and no NAA (medium 11) after 4 weeks in culture.


8
11

This multiplication rate could be acceptable commercially compared with the three-fold multiplication rate obtained per month with 'Improved Blaze' rose on MS basal medium containing BAP (3.0 mg/liter), IAA (0.3 mg/liter) and bacto-agar (8 g/liter) (Hasegawa, 1979). Shoot multiplication of different rose species or cultivars was obtained with either low or high BAP levels. Shoot proliferation of 'Forever Yours' rose was achieved using a MS basal medium with BAP and NAA at the rates of 2.0 and 0.1 mg/liter respectively (Skirvin and Chu, 1979). Shoot multiplication of seven rose cultivars (Davies, 1980), two new and two old world rose species (Khosh-Khui and Sink, 1982c) and 'Forever Yours' rose (Skirvin and Chu, 1979) was inhibited at BAP concentrations higher than 2.0 mg/liter. In contrast, BAP at concentrations of 1.0, 3.0 and 10.0 mg/liter improved shoot multiplication of 'Improved Blaze' rose (Hasegawa, 1980).

Shoot multiplication of 'D.K.' rose varied with different concentrations of BAP and NAA in the shoot multiplication medium (Figure 3.5). Media with 0 or 5.0 mg/liter BAP did not promote any new shoot development. The existing shoots started to yellow and soon deteriorated. Two to three shoots formed in media 6-8 and 17-20 while three to four shoots formed in media 11-15.

Figure 3.5 The effects of MS basal medium supplemented with various BAP and NAA concentrations (mg/liter) on shoot multiplication of 'D.K.' rose after 4 weeks in culture.



The lack of BAP, low BAP:NAA ratio or BAP levels higher than 2.5 mg/liter in the culture medium negatively affected shoot multiplication of 'D.K.' rose. The same was observed with thirty six dwarf rose cultivars including 'D.K.' cultured on a shoot multiplication medium consisting of MS basal medium supplemented with 1 mg/liter BAP and 0.1 mg/liter GA_3 (Dubois et al., 1988). The best media for shoot multiplication of 'D.K.' roses had BAP:NAA concentrations of 0.1:0.25 (medium 9) and 0.1:0.5 mg/liter (medium 10); both media promoted the formation of eight to ten shoots per explant (Figure 3.6). This multiplication rate is acceptable for commercial rose micropropagation.

These results showed that the establishment of nodal stem segments and shoot multiplication of 'Q.E.' and 'D.K.' roses could be successfully obtained with low rates of BAP (0.1-1.0 mg/liter) and NAA (0-0.5 mg/liter) with a seven-ten fold monthly multiplication rate. These results are consistent with previous findings where variation in the response to growth regulator levels in the multiplication medium was found among plant species. The optimal growth regulator concentrations in the culture medium should be determined from examination of a wide range of cytokinin:auxin combinations.

Figure 3.6 Shoot multiplication of 'D.K.' rose on MS basal medium supplemented with 0.1 mg/liter BAP and 0.25 mg/liter NAA (medium 9) or with 0.1 mg/liter BAP and 0.5 mg/liter NAA (medium 10) after 4 weeks in culture.


9
10

3.3.2 Experiment 2

Lateral shoots of 'Q.E.' subcultured to shoot multiplication medium after bud break produced six to seven new axillary shoots after 40 days. Those placed into root initiation medium developed four to seven roots each after 2 weeks (Figure 3.7). Lateral shoots of 'D.K.' subcultured into shoot multiplication medium produced three to four new shoots after 40 days. Those placed into the rooting medium yellowed, senesced and did not form any root initials within 2 weeks. These results do not support those of Dubois *et al.* (1988) who reported "successful" (success rate was not included) root initiation of 'D.K.' rose, among thirty five other dwarf rose cultivars tested.

Plantlets of 'Q.E.' did not survive after *ex vitro* transplantation. Transplant failure is not uncommon for woody species and was probably due to the relatively low light intensity in the growth chamber (55.44 $\mu mol.m^{-2}.s^{-1}$). Subsequent transplantation trials were successfully achieved at a light intensity of 124.74 $\mu mol.m^{-2}.s^{-1}$ (Chapter V; section 5.3.1). It is generally recognized that freshly transplanted plantlets show greater growth and higher survival rates if they are initially placed under low light intensities and gradually moved to higher light intensities (60-130 $\mu mol.m^{-2}.s^{-1}$) (Connor and Thomas, 1981).

Figure 3.7 Plantlets of 'Queen Elizabeth' rose after 14 days in the rooting medium consisting of MS basal medium containing 1 mg/liter IBA.

However, this was not true of roses in this experiment, nor was it true for red raspberry (Rubus idaeus L.) where optimal light intensity ex vitro was about three-fold higher than in vitro (Donnelly and Vidaver, 1984a; Donnelly and Vidaver, 1984b). Transplantation to soil of various rose cultivars was successful (60-80 % survival) under a photon flux density (400-700 nm) of 66 $\mu mol.m^{-2}.s^{-1}$ for 12-24 hr daily (Bressan et al., 1982). The establishment of 'Improved Blaze' rose in soil was higher (90-100 %) under higher light intensities (126 $\mu mol.m^{-2}.s^{-1}$) (Hasegawa, 1980).

3.4 Summary and Conclusion:

Nodal stem explants of 'Q.E.' and 'D.K.' roses were successfully established and the resulting shoots multiplied on MS basal medium supplemented with low rates of BAP (0.1-1.0 mg/liter) and NAA (0-0.5 mg/liter). A seven-ten fold multiplication rate per month was obtained for both rose cultivars compared with a three-fold multiplication rate obtained with 'Improved Blaze' rose (Hasegawa, 1979). Ascorbic and citric acids (50 and 75 mg/liter, respectively) in the media, as recommended by Dr. Arnold, prevented the production of phenols and allowed shoot and plantlet growth of 'Q.E.' and 'D.K.' roses. Root initia-

tion of 'Q.E.' but not 'D.K.' shoots occurred after 2 weeks on MS basal medium with 1 mg/liter IBA. Ex vitro transplantation of 'Q.E.' plantlets was not successful and was attributed to the low light intensity of the transplant environment.

These trials established efficient protocols for the explant establishment and shoot multiplication of 'Q.E.' and 'D.K.' roses. Although root initiation of 'Q.E.' rose was achieved, further studies are needed to assess the appropriate nutrient medium requirements for the root initiation of 'D.K.' rose. These could include testing: (1) reduced concentrations of MS basal salts, including one-half or one-quarter strength (Hasegawa, 1980; Skirvin and Chu, 1979), (2) other types and concentrations of auxins, (3) various auxin:cytokinin combinations and (4) decreased night temperatures (Bressan et al., 1982; Khosh-Khui and Sink, 1982b).

Micropropagation protocols for roses seem to be species and cultivar-dependent. There is always a need to develop specialized culture media for desirable or valuable cultivars. As new information is reported, the procedures should be altered to improve the propagation and rooting efficiency. Finally, only 'Q.E.' rose was selected for the shaking stress treatments because root initiation of 'D.K.' rose shoots was not achieved and the greenhouse-grown 'D.K.' roses were lost due to disease.

CHAPTER IV

GREENHOUSE RESEARCH EXPERIMENTS

4. THE EFFECTS OF MIS ON IN VIVO ROSE CUTTINGS:

4.1 Introduction:

Commercially, bedding plants are hardened-off (conditioned) prior to transplantation into the field by gradually reducing the ambient temperature or water or both and the nutrient availability. MIS treatment was useful to harden-off vegetable seedlings such as lettuce, cauliflower and celery to withstand physical or physiological damage that might occur during or after transplantation (Biddington and Dearman, 1985a). Automated shaking stress was used to control the height of chrysanthemum without the use of expensive or polluting chemicals. The nature of these growth retarding chemicals was not defined (Beyl and Mitchell, 1977b). The advantages of MIS included shoot strengthening, ready reversibility of growth retardation and automation (Beyl and Mitchell, 1977a).

The primary objective of these experiments was to determine whether MIS (shaking stress) was useful in improving the early establishment of conventionally-propagated rose cuttings. Experiments were carried out to characterize the effects of MIS on the performance of 'Q.E.' and 'D.K.' rose cuttings during rooting in a mist chamber and on rooted rose cuttings after transfer from a mist chamber to a growth chamber. Plant size, growth rates and dry matter accumulation of various organs were measured following the treatments. Anatomical evaluations of mechanical tissue formation such as collenchyma, sclerenchyma and cell wall thickness in various organs would be carried out only when morphological changes or dry matter accumulation were affected by the treatments. A secondary objective, that emerged during the course of these experiments, was to investigate the pathogen(s) implicated in causing the death of the rose plants in the mist and the growth chambers.

4.2 Materials and Methods:

4.2.1 MIS experiments

Semi-hardwood cuttings, 2-4 cm long, consisting of a single node with a leaflet and a dormant bud of 'Q.E' and

'D.K.' roses were dipped in Stim-Root #3 [R] rooting hormone powder (0.8 % IBA) prior to individual insertion into twenty four-cell germination flats (Kord [R]). The germination flats contained a potting mixture consisting of 1 perlite : 1 vermiculite : 1 Pro-Mix BX [R] (by volume) amended with 10 g each of dolomitic limestone and 19N-6P_2O_5-12K_2O Osmocote [R] per liter of mixture, and watered with an aqueous solution of 10N-52P_2O_5-10K_2O transplanting fertilizer (5 g/liter). Lesan 35 % WP [R] (Fenaminosulf), a soil drench fungicide, was incorporated into the rooting medium at the rate of 1 g/liter H_2O. Benlate [R] and Truban [R] were sprayed weekly at the rates of 0.5 g/liter H_2O. A foliar fertilizer 27N-9P_2O_5-12K_2O was applied weekly at the rate of 3.5 g/liter H_2O. The flats were placed in a mist chamber with bottom heat (25-27 ^oC) and misting nozzles which released water every 10 min for 30 sec, from 8:00 am to 5:00 pm.

After 4 weeks in the mist chamber the rooted cuttings were transferred singly into twenty four-cell flats containing only pasteurized Pro-Mix BX [R] with the same amendments (dolomitic limestone and Osmocote [R]), and irrigated with 20N-20P_2O_5-20K_2O water soluble fertilizer at the rate of 3 g/liter H_2O. At transfer time and one week later, the rooted cuttings were sprayed with the Benlate [R] and Truban [R] fungicide mixture. The flats were covered with clear plastic lids and placed in a clean growth

chamber set at 25 ± 1 ^{o}C, with a 16 hr photoperiod and an average relative humidity of 40 %. Each shelf held two flats illuminated by cool white fluorescent lamps (20 W) providing an average light intensity of 66.28 $\mu mol.m^{-2}.s^{-1}$ at plant height. The plastic covers were lifted gradually over a period of 2 weeks. This experiment was repeated three times.

Shaking treatments were started for cuttings of both rose cultivars during rooting in the mist chamber (rooting stage) and when rooted cuttings were transferred from the mist chamber to the growth chamber (acclimatization stage). Each treatment group consisted of twenty four plants in a twenty four-cell germination flat. The flats were arranged in a completely randomized design in both the mist chamber and the growth chamber. Flats were placed singly on an oscillatory orbit shaker frame (Lab Line ^{R}, Model 3520) and were either not shaken (treatment 0) or shaken daily at 150 rpm for 15 min (treatment 1) or 30 min (treatment 2) or at 200 rpm for 15 min (treatment 3) or 30 min (treatment 4) for 4 weeks.

The fresh weights and the stem and petiole diameters of all cuttings for rooting and acclimatization stages (treatments 0-4) of both rose varieties were initially recorded. When rooting stage treatments were completed, rooted cuttings were evaluated for fresh weights, number and length of roots and stem and petiole

diameters. Stem and petiole diameters were measured on the same cuttings since bud break and new shoot development had not occurred in 'Q.E.' or it did in 'D.K.' rose but the new growing shoots were too small to be measured after 4 weeks of treatment. Also, one-quarter of surviving rooted cuttings, from both rose cultivars of each treatment, were randomly selected and harvested at the end of the rooting stage for shoot and root fresh and dry weights. Dry weights were obtained by drying shoots and roots in Petri dishes in a 60 ^{o}C oven for 3 days. After 2 weeks in the growth chamber all 'Q.E.' and 'D.K.' plants died due to disease (Section 4.2.2) so, final data could not be recorded.

Calculated parameters for rooting stage plants included specific shoot (SSWC) and root (SRWC) water content (water weight per tissue dry weight), percent shoot (PSDM) and root (PRDM) dry matter (shoot or root dry weight/ shoot or root fresh weight) and root:shoot dry weight ratios. Data collected on the rooting stage of 'Q.E.' and 'D.K.' roses were subjected to analysis of variance (General Linear Models Procedure; Appendix III). Mean separation of treatments was tested using the Duncan's multiple range test at the 5 % level of significance.

At the end of the rooting stage, plant material was not collected to observe for differences in internal

anatomy between mechanically-shaken and non-shaken in vivo rose cuttings because only new roots had formed at this point. Sampling was scheduled for the end of the acclimatization stage. However, since rose plants were repeatedly lost to disease during acclimatization in the growth chamber, sampling was not done and consequently, anatomical evaluations were not carried out.

4.2.2 Pathogen isolations

Rose plants were lost to disease in all three trials of these experiments. Poor survival was thought to be due to an infection caused by a pathogen. To isolate this pathogen infected cuttings from each trial were randomly selected from the mist and growth chambers and ranged from mildly infected (decayed shoot apex) to severely infected (completely decayed). After surface-sterilization in 10 % Javex ^{R} for 5 min and washing in double distilled, sterile water, small pieces were aseptically plated on Potato Dextrose Agar (PDA; 6 g/liter H_2O) in Petri dishes and incubated in a completely randomized design under cool white fluorescent lights (45 $\mu mol.m^{-2}.s^{-1}$) and a 16 hr photoperiod for 1 week at 25 $^{o}C \pm 1\ ^{o}C$. Pro-Mix BX ^{R}, Stim-Root #3 ^{R} and cuttings from the greenhouse-grown stock plants, suspected to be possible sources of infection, were aseptically plated on PDA and incubated under the same

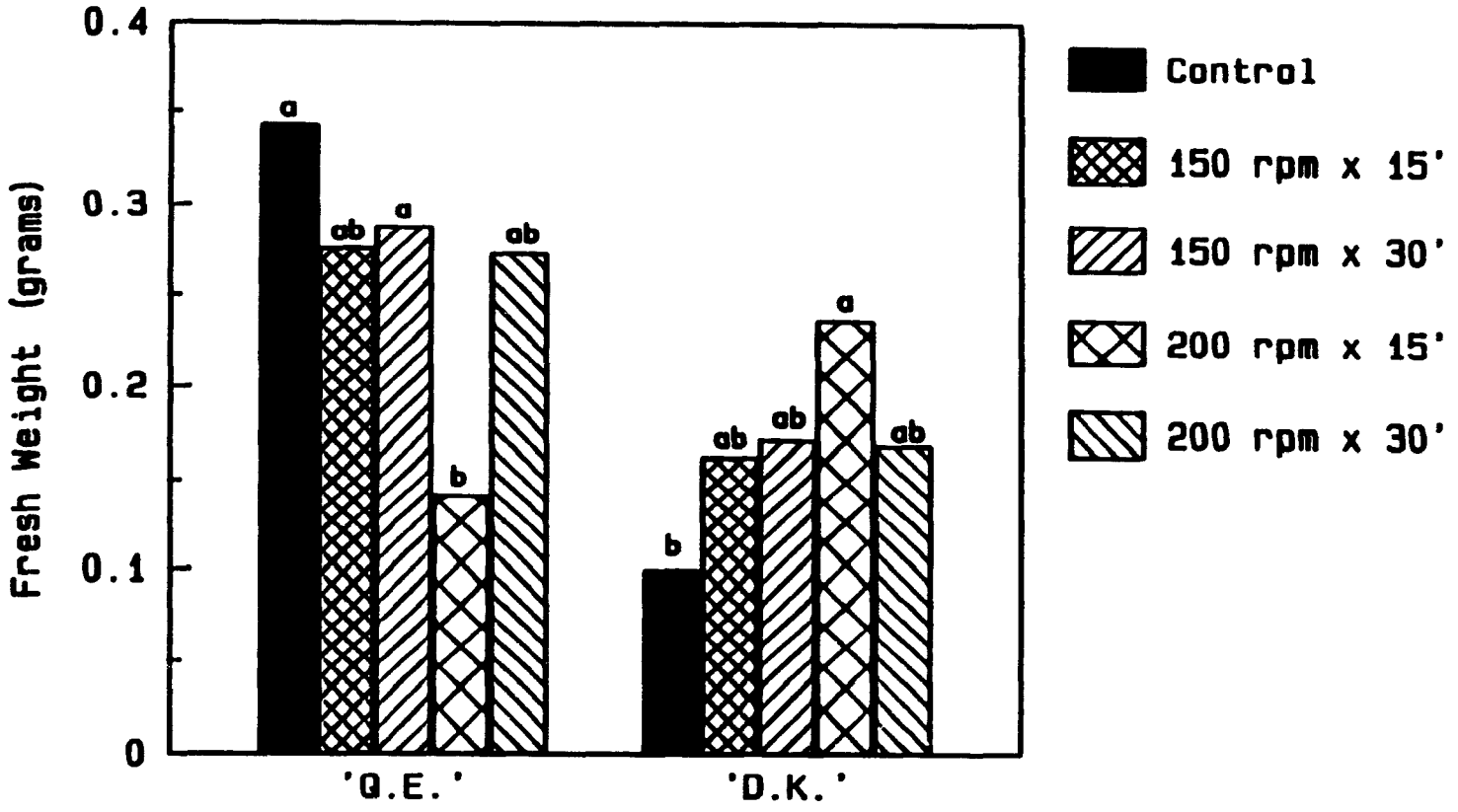
conditions. There were three plates/treatment. The mean number of infected cuttings per plate and treatment was recorded. Fungal mycelial growth from each plate and treatment was microscopically observed and identified one week later.

4.3 Results and Discussion:

4.3.1 MIS experiments

Only the results of the effects of shaking stress on 'Q.E.' and 'D.K.' roses harvested after rooting in the mist chamber will be discussed as rooted rose cuttings were lost to disease during acclimatization. The change in total fresh weight was significantly less in 'Q.E.' and more in 'D.K.' rose cuttings in treatment 3 compared with non-shaken control cuttings (Figure 4.1). Growth retardation of 'Q.E.' rose cuttings caused by shaking is a typical response to MIS (Jaffe, 1973; Jaffe, 1976a; Akers and Mitchell, 1984). However, the opposite response of 'D.K.' rose cuttings is possibly species-related.

Figure 4.1 The effect of daily shaking for 4 weeks during the rooting stage on the change in total fresh weight of 'Q.E. and 'D.K.' rose cuttings. Different upper case letters indicate mean separation of treatments (Duncan's multiple range test, 5 % level).


0.4
0.3
0.2
0.1
0
Fresh Weight (grams)
a
ab
a
b
ab
b
ab
ab
a
ab
'Q.E.'
'D.K.'
Control
150 rpm x 15'
150 rpm x 30'
200 rpm x 15'
200 rpm x 30'

Shoot fresh and dry weights of MIS-treated 'Q.E.' rose cuttings were not altered relative to undisturbed control cuttings as bud break had not yet occurred in these cuttings after 4 weeks of treatment (Table 4.1). However, shoot fresh weight of 'D.K.' rose cuttings was significantly increased by treatment 4 and shoot dry weight was significantly increased by treatments 3 and 4 compared with control cuttings (Table 4.1). It must be noted that bud break occurred in 'D.K.' rose cuttings and shoots started to develop.

The reaction of woody plants to MIS is not well documented yet. The increase in shoot fresh and dry weight of 'D.K.' rose cuttings after shaking compared with the control cuttings might also be due to the difference in plant species. Shoot fresh and dry weights of many herbaceous plant species were significantly reduced after MIS treatments (Biddington and Dearman, 1985a; Heuchert and Mitchell, 1983; Mitchell et al., 1977; Beyl and Mitchell, 1977b; Latimer et al., 1986). However, the extent of weight reductions differed among plant species. For example, bryony displayed a considerable shoot weight reduction, tomato an intermediate amount and pea none at all. Also two taxonomically related plants, cucumber and pumpkin, reacted completely differently to MIS (Jaffe, 1973).

Table 4.1 The effect of daily shaking for 4 weeks during the rooting stage on shoot and root fresh and dry weight (Wt) of 'Q.E.'and 'D.K.' rose cuttings.

Treatment	Shoot		Root	
	Fresh Wt (g)	Dry Wt (g)	Fresh Wt (g)	Dry Wt (g)
'Q.E.'				
0 Control	0.676ab[z]	0.141ab	0.217ab	0.016bc
1 150 rpm X 15'	0.918a	0.212a	0.150b	0.011bc
2 150 rpm X 30'	0.490b	0.115b	0.180b	0.022b
3 200 rpm X 15'	0.473b	0.103b	0.050b	0.005c
4 200 rpm X 30'	0.590b	0.100b	0.370a	0.040a
'D.K.'				
0 Control	0.142b	0.027b	0.029a	0.002b
1 150 rpm X 15'	0.217ab	0.035ab	0.062a	0.004ab
2 150 rpm X 30'	0.220ab	0.047ab	0.057a	0.004ab
3 200 rpm X 15'	0.237ab	0.057a	0.062a	0.007a
4 200 rpm X 30'	0.283a	0.060a	0.053a	0.006ab

z Mean separation of treatments (Duncan's multiple range test at the 5 % level)

Root fresh weights of shaken 'Q.E.' and 'D.K.' roses were not altered compared with undisturbed controls. However, root dry weights of 'Q.E.' and 'D.K.' roses were significantly greater following treatments 4 and 3, respectively (Table 4.1).

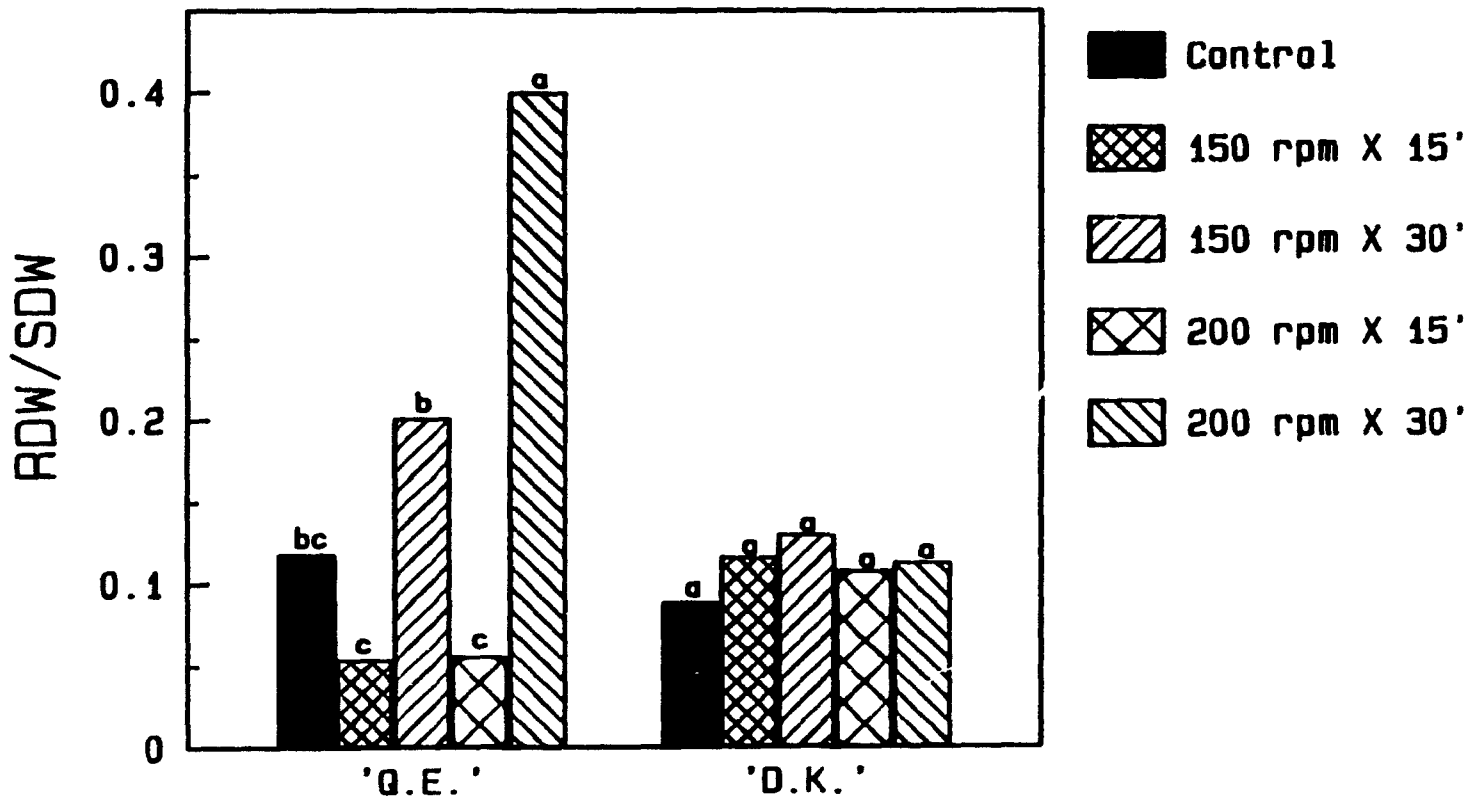
Not all effects of MIS were the same between plant species. A significant reduction in root dry weight was observed in lettuce, cauliflower and celery seedlings after brushing (Biddington and Dearman, 1985a). However, there was no effect on the root weight after "handling" of pumpkin petioles and leaves (Turgeon and Webb, 1971). Similar variation in responses were observed between the

two rose species.

The root:shoot dry weight ratio of MIS-treated 'Q.E.' rose cuttings was significantly greater in treatment 4 relative to non-treated controls and not altered in 'D.K.' roses (Figure 4.2). The increased ratio of root:shoot dry weight in treatment 4 for 'Q.E.' rose cuttings suggests a dry matter accumulation in the roots relative to the shoots. This is further supported by the significant increase in root dry weight reported earlier for 'Q.E.' rose cuttings after similar treatment. It is possible that the increase in root and shoot dry weights of 'D.K.' rose cuttings reported earlier after treatment 3 was proportional resulting in no significant change in the root:shoot dry weight ratio.

Different responses in the ratio of root:shoot dry weight were also reported for herbaceous species. The ratio of root:shoot dry weight was increased in brushed lettuce but reduced in celery and unaltered in cauliflower seedlings (Biddington and Dearman, 1985a). The development of a larger root system at the expense of shoots usually occurs under water stress or dry conditions because the evaporative demand of the shoot becomes low and the relatively larger root system is better able to exploit the limited water supply (Biddington and Dearman, 1985a). This hypothesis is not applicable to the present experiments since the rose cuttings were kept in mist chambers.

Figure 4.2 The effect of daily shaking for 4 weeks during the rooting stage on the ratio of root (RDW):shoot (SDW) dry weight of 'Q.E.' and 'D.K' rose cuttings. Different upper case letters indicate mean separation of treatments (Duncan's multiple range test, 5 % level).


RDW/SDW
0.4
0.3
0.2
0.1
0
bc
c
b
c
a
a
a
a
a
a
'Q.E.'
'D.K.'
Control
150 rpm X 15'
150 rpm X 30'
200 rpm X 15'
200 rpm X 30'

Seismic stress did not affect root number of either rose cultivar but it significantly enhanced root elongation in 'Q.E.' rose cuttings in treatment 4 and in 'D.K.' roses in treatments 1, 2 and 3 compared with non-shaken controls (Table 4.2). Elongation of the root system of 'Q.E.' rose cuttings in response to shaking at 200 rpm for 30 min daily for 4 weeks directly influenced the root dry weight and subsequent root:shoot dry weight ratio. The same applies for 'D.K.' rose cuttings shaken at 200 rpm for 15 min which increased root dry weight due to the increased root elongation.

In contrast, it was reported that brushing reduced root length of cauliflower, lettuce and celery seedlings. However, the ratio of total root length to the number of lateral roots was similar in control seedlings. Thus, it was noted that the pattern of root growth was not altered by mechanical stimulation (Biddington and Dearman, 1985a). Although treatments 1 and 2 promoted root elongation of 'D.K.' rose cuttings without causing any significant changes in the root fresh or dry weights, this increase in root length did not result in a change in the overall pattern of root growth.

Table 4.2 The effect of daily shaking for 4 weeks during the rooting stage on the change in petiole and stem diameter (diam.) and on root number (no.) and length (L) of 'Q.E.'and 'D.K.' rose cuttings.

Treatment	Petiole diam.(cm)	Stem diam.(cm)	Root no.	Root L. (cm)
'Q.E.'				
0 Control	0.017a[z]	0.037a	5.41a	2.39b
1 150 rpm X 15'	0.017a	0.017a	5.85a	2.56b
2 150 rpm X 30'	0.018a	0.015a	5.70a	2.86ab
3 200 rpm X 15'	0.022a	0.034a	4.35a	2.44b
4 200 rpm X 30'	0.018a	0.016a	5.62a	3.41a
'D.K.'				
0 Control	0.008a	0.004b	4.41a	2.05c
1 150 rpm X 15'	0.009a	0.016a	8.73a	3.52ab
2 150 rpm X 30'	0.013a	0.011ab	6.06a	3.83a
3 200 rpm X 15'	0.012a	0.005b	6.35a	3.63ab
4 200 rpm X 30'	0.004a	0.001b	4.50a	2.63bc

[z] Mean separation of treatments (Duncan's multiple range test at the 5 % level)

Petiole diameters were not altered following shaking of 'Q.E.' and 'D.K.' rose cuttings relative to undisturbed controls (Table 4.2). In contrast, petiole diameters significantly increased in pumpkin seedlings after gentle shaking for 30 sec daily for 20 days (Turgeon and Webb, 1971).

Stem diameters did not change in 'Q.E.' rose but significantly increased in 'D.K.' rose cuttings in treatment 1 (Table 4.2). Stem diameters were also increased following rubbing of bean (Jaffe et al., 1984) and tomato (Heuchert et al., 1983) but were reduced in soybean

(Glycine max L.) (Latimer et al., 1986). Stem swelling in response to thigmic stress implicated ethylene since the severity of the MIS treatment and the subsequent response of plants is mediated by the amount of wound ethylene produced (Biro et al., 1980).

The specific shoot and root water content of 'Q.E.' and 'D.K.' rose cuttings were not altered after shaking compared with non-shaken control cuttings (Table 4.3). Again, these results with roses are in contrast to reports with herbaceous species where thigmic or seismic stress significantly reduced SSWC of soybean and eggplant in the greenhouse and outdoor environments (Latimer et al., 1986). Also, SSWC was reduced when tomato seedlings were shaken daily for 22 days at 175 rpm for 5 min in winter but not in summer, and it decreased progressively as the shaking rate increased from 125 rpm to 175 rpm for 20 min daily for 22 days compared with undisturbed control seedlings (Heuchert and Mitchell, 1983).

The percent shoot dry matter of both rose varieties was not altered after shaking. The percent root dry matter was significantly increased in 'Q.E.' rose cuttings in treatment 2 compared with undisturbed controls, suggesting an increase in root dry weight relative to root fresh weight but it did not change in 'D.K.' rose (Table 4.3).

Table 4.3 The effect of daily shaking for 4 weeks during the rooting stage on the specific shoot (SSWC) and root (SRWC) water content and the percent shoot (PSDM) and root (PRDM) dry matter of 'Q.E.'and 'D.K.' rose cuttings.

Treatment	Shoot		Root	
	SSWC (g H_2O g dry wt^{-1})	PSDM (%)	SRWC (g H_2O g dry wt^{-1})	PRDM (%)
'Q.E.'				
0 Control	3.94a[z]	20.90a	12.21a	7.77b
1 150 rpm X 15'	3.46a	22.92a	14.00a	7.40b
2 150 rpm X 30'	3.28a	23.39a	7.37a	13.54a
3 200 rpm X 15'	4.32a	21.06a	9.00a	10.00ab
4 200 rpm X 30'	4.90a	16.94a	8.25a	10.81ab
'D.K.'				
0 Control	4.65a	18.94ab	13.93a	7.90ab
1 150 rpm X 15'	5.25a	16.08b	17.58a	6.65b
2 150 rpm X 30'	4.71a	21.30ab	12.35a	7.64ab
3 200 rpm X 15'	3.08a	25.46a	11.16a	13.15a
4 200 rpm X 30'	4.00a	20.63ab	7.80a	12.90ab

z Mean separation of treatments (Duncan's multiple range test at the 5 % level)

4.3.2 Pathogen isolations

Plated cuttings from the first acclimatization trial were infected by Pythium sp. The possible reasons for infection were the use of (1) unpasteurized potting mixture Pro-Mix BX ^{R}, a potential source of various fungal contaminants and (2) an ineffective fungicide mixture consisting of Benlate ^{R} and Truban ^{R}. Benlate 50 WP ^{R} was used as it is recommended in the control of Fusarium, Botrytis and powdery mildew fungi on various crops.

Truban [R] was combined with Benlate [R] as it is effective in controlling damping-off and root and stem rot diseases caused by Pythium and Phytophthora spp.

In the second acclimatization trial, isolated cuttings from the mist chambers and the growth chambers were infected by Fusarium sp.. Plated pasteurized Pro-Mix BX [R] and rooting hormone powder did not show any fungal growth and were eliminated as possible sources of infection. Cuttings from the greenhouse stock plants showed fungal mycelial growth (one cutting/plate/treatment) which was identified as a Fusarium sp. Therefore, the infected stock plants were apparently the cause of the subsequent infection.

In an attempt to control the Fusarium pathogen, Lesan 35 WP [R], a soil drench fungicide, was incorporated into the rooting medium for the third trial and cuttings were sprayed with Benlate [R] and Truban [R]. However, disease protection and control was unsuccessful and plated rose cuttings isolated from the mist chambers and the growth chambers were again infected with Fusarium sp.. The Fusarium contamination was difficult to control in this experiment even with appropriate use of recommended fungicides because the source plants were infected and the spread of the pathogen was further enhanced by the large wounds of the cuttings and the high relative humidity in the mist chamber. This resulted in the forced discontinua-

tion of the acclimatization of 'Q.E.' and 'D.K.' roses.

4.4 Summary and Conclusion:

Morphological changes caused by mechanical stress contribute to the survival of plants as one of their adaptive strategies under severe enviro l conditions such as drought or saline stress (Suge, .980). Mechanical perturbation enhanced plant strength and hardiness by reducing apical growth and increasing lateral growth (Heuchert and Mitchell, 1983).

In these experiments, shaking 'Q.E.' rose cuttings at 200 rpm for 30 min daily for 4 weeks during the rooting stage increased root dry weight, root to shoot dry weight ratio and root length. Shaking 'D.K.' rose cuttings at 200 rpm for 15 min increased their shoot fresh and dry weight, root dry weight and root length. These results suggested that shaking stress improved rooting. However, it is worth noting that most grow'h parameters, measured or calculated, had a contrary response to mechanical stress compared with MIS-treated herbaceous plants mentioned in the literature. The possible reasons for such responses could be differently interpreted. Two components of shaking action have been identified on plant growth and involve (a) the physical stress resulting from bending or

displacement of plant tissues and (b) alteration of leaf microclimate and gas exchange. Growth reduction may result from both components of shaking action, but the relative contribution of each has not been determined yet (Grace and Thompson, 1973). This was further supported by Grace et al. (1982) who explained that the response to shaking involves the following steps: (a) opening of stomata, (b) increased transpiration rate caused by open stomata, (c) decreased water potential in response to the increased transpiration rate and (d) reduced extension growth caused by the lower water potential. Although daily shaking of 'Q.E.' and 'D.K.' roses might have triggered the two components mentioned, the high humidity level in the mist chamber would have controlled the transpiration rate and possibly nullified the shaking effect.

The differential responses of 'Q.E.' and 'D.K.' roses could be species dependent. Variation among species and cultivars in response to mechanical perturbation has been previously reported (Jaffe, 1973; Beyl and Mitchell, 1977a; Akers and Mitchell, 1984; Biddington and Dearman, 1985a; Latimer et al., 1986). This variation has been attributed to the differences in growth form or habit or other inherent characteristics. Although there is no clear reason why species respond differently to MIS, interactions and interdependences between the many responses, some or even most of which may be secondary, may account for some

of these differences (Biddington, 1985a).

Daily and seasonal variation in dosage of light intercepted, spectral quality of solar radiation and relative humidity are among the uncontrollable variables that may determine the response of plants to mechanical stress in the greenhouse (Heuchert and Mitchell, 1983). For this reason, Akers and Mitchell (1984) recommended that plant growth responses to MIS must be studied in protected and controlled environments. This was attempted in the present experiments.

In conclusion, it is not unusual to get large mortality rates when propagating roses through cuttings. Shaking stress was tested to determine whether it could impart strength and hardiness to roses after rooting and promote early establishment and growth. Unfortunately, long term evaluations of shaking stress on rose cuttings were discontinued due to Fusarium disease.

CHAPTER V

TISSUE CULTURE RESEARCH EXPERIMENTS

5. THE EFFECTS OF MIS ON IN VITRO ROSES:

5.1 Introduction:

The commercial production of micropropagated plantlets is often limited by poor survival when plantlets are transferred to soil. Tissue-cultured plantlets are difficult to transplant for four main reasons: (1) they grow mixotrophically or heterotrophically in culture and must become fully autotrophic after transplantation, (2) they grow under conditions of almost 100 % relative humidity with poor gas exchange resulting from the closed containers and therefore tend to have a reduced ability to control water loss after transplantation to lower relative humidity environments (Murashige, 1977), (3) they are tender and fragile, partly because of the high relative humidity and relatively low light intensity in culture and (4) they develop little support tissue presumably because of the lack of wind or air turbulence in stationary

cultures (Donnelly et al., 1985).

Many controlled hardening-off procedures have been used to improve ex vitro survival of micropropagated plantlets. Increasing the agar or sucrose concentration in the culture medium (Connor and Thomas, 1981; Murashige, 1977) and the light intensity in the culture environment (Lee et al., 1988) during the rooting stage improved the tolerance of plantlets to moisture stress after transplantation. Pre-exposure to low temperatures enhanced root development of roses and subsequent transplantation to soil (Bressan et al., 1982; Khosh-Khui and Sink, 1982b). Gradual acclimatization periods of decreasing relative humidity in the culture vessels were used to improve plantlet survival when transferred from culture to soil (Brainerd and Fuchigami, 1981; Wardle et al., 1983).

Morphological changes such as reduced stem and petiole elongation, leaf weight and area and increased stem diameter in response to MIS resulted in small and compact plants with increased strength and hardiness. Such changes were interpreted as adaptive responses which enabled the plants to resist physical stress (Heuchert et al., 1983; Biddington and Dearman, 1985a; Biddington, 1985a). In addition, an increase in strengthening tissue was reported in plants exposed to wind. Mechanical stress in the form of wind motion stimulated the development of more extensive collenchyma deposition with heavier cell wall thickening

and larger cell diameters in celery petioles (Venning, 1949). The sclerenchymatous leaf margin of wind-exposed corn (Zea mays L.) was six times as wide as in the control leaves (Whitehead and Luti, 1961). Thorn-apple plants which were subjected to mechanical shaking for 9 hrs daily for 40 days produced more heavily thickened collenchyma cell walls compared with control plants, but MIS inhibited collenchyma cell elongation (Walker, 1960).

The primary objective of these experiments was to determine whether MIS treatment was useful in improving ex vitro transplantation, survival and hardiness of 'Q.E.' rose plantlets by increasing strengthening tissue formation. The effects of shaking stress applied at the shoot multiplication and the rooting stages were evaluated. This included an examination of plant size, growth rates and dry matter accumulation of various organs. The secondary objective was to microscopically evaluate mechanical tissue formation to determine whether it was affected by MIS treatment. So, collenchyma, sclerenchyma and cell wall thickness were examined in roots, stems, leaves and petioles of mechanically-stressed and non-stressed tissue-cultured plantlets. In addition, the internal anatomy of control in vivo and in vitro 'Q.E.' plants was examined.

5.2 Materials and Methods:

5.2.1 MIS experiments

Stem sections with several nodes (10-12 cm long, with 2-4 buds) of greenhouse-grown 'Q.E.' roses were surface-sterilized in 10 % Javex ^{R} for 20 min and rinsed three times in sterile, double distilled water. Single node segments, 2-3 cm long with one lateral bud, were aseptically excised and transferred to a shoot multiplication medium consisting of MS basal salts with 1 mg/liter BAP and 0.1 mg/liter GA_3. The medium was adjusted to pH 5.8 prior to Anachemia ^{R} agar addition (6 g/liter), dispensed into 25 X 150 mm test tubes (10 ml/test tube) and autoclaved at 121 ^{o}C, 1.055 kg.cm^{-2} for 20 min.

Cultures, in racks of twenty four test tubes, were incubated in a culture room set at 25 ^{o}C $\pm$ 1 ^{o}C with a 16 hr photoperiod under cool white fluorescent lamps (40 W) providing 55.95 μmol.m^{-2}.s^{-1} at culture level. Explants underwent bud break and were kept in the shoot multiplication medium for 4 weeks. Shoots were then aseptically severed from the original stem portion and transferred to a rooting medium consisting of MS basal salts with 1 mg/liter IBA and no BAP or GA_3. Two weeks later, plantlets were rinsed to remove the agar from the roots. Roots were drenched in a fungicide mixture consisting of Benlate ^{R} and

Truban ^{R} (0.5 g/liter H_2O) prior to transplantation. All plantlets were then randomly transplanted into pasteurized Pro-Mix Bx ^{R} drenched with the fungicide Subdue 2G ^{R} (Metalaxyl) (125 gm/m^3 Pro-Mix BX), amended with dolomitic limestone and 14N-14P_2O_5-14K_2O Osmocote ^{R} (10 g and 6 g/liter Pro-Mix BX ^{R}, respectively) and contained in forty eight-cell germination flats (Kord ^{R}). After planting and one week later, the potting mixture was irrigated with a soluble fertilizer 20N-20P_2O_5-20K_2O at the rate of 3 g/liter H_2O.

The plastic-covered flats were placed in a growth chamber at 25 ^{o}C $\pm$ 1 ^{o}C with a light intensity of 55.4 μmol.m^{-2}.s^{-1} (first 2 trials) or 124.74 μmol.m^{-2}.s^{-1} (third trial) provided by cool white fluorescent lamps with a 16 hr photoperiod for a 5 week period. After one week, the plastic covers were gradually lifted over a 2 week interval.

Shaking treatments started either: (1) at the shoot multiplication stage (on single node explants which underwent bud break and subsequent shoot development), lasting 4 weeks, followed by transfer to the rooting medium and subsequent acclimatization in a growth chamber for 5 weeks or (2) at the rooting stage (on control severed shoots derived from the shoot multiplication stage), lasting 2 weeks, followed by acclimatization in a growth chamber for 5 weeks.

Each treatment group consisted of twenty four (1) single node explants or (2) severed shoots placed singly in test tubes held in racks arranged in a completely randomized design on the shelves of the culture room. Control plants were non-shaken plants (treatment 0). Shaking treatments involved placing racks daily on an oscillatory orbit shaker frame, and shaking them at 150 rpm for 15 min (treatment 1) or 30 min (treatment 2) or at 200 rpm for 15 min (treatment 3) or 30 min (treatment 4).

Initial weight of the explants was recorded by weighing the test tubes prior to and after aseptic insertion of the explant. In the same way, the weight of the severed shoots transferred to the rooting medium was also recorded. Leaf number per shoot was recorded at the end of the shoot multiplication stage. At the same time, a subsample consisting of four plants from treatments 0-4 were used to measure shoot fresh and dry weights and stem lengths. At the end of the rooting stage and at the end of the acclimatization stage, the following growth parameters were measured for all treatments: plantlet fresh weight, leaf and root number/plantlet, stem length and diameter and root length. At the end of the rooting stage, four plantlets from treatments 0-4 were randomly selected for shoot and root dry weight measurements. Samples were dried in an oven at 60 ^{o}C for 3 days. Calculated parameters included SSWC, SRWC, PSDM, PRDM and root:shoot dry weight

ratio. Data collected from the third trial were subjected to analysis of variance (General Linear Models Procedure; Appendix IV). Mean separation of treatments was tested using the Duncan's multiple range test at the 5 % level of significance. From the first two trials, data were collected up to the time of transplantation to soil and were similarly analysed.

5.2.2 Internal anatomy

At the end of the shoot multiplication and the rooting stages, two shoots or plantlets were randomly selected from each treatment for anatomical evaluation, along with two samples from mature greenhouse-grown control 'Q.E.' rose plants. Petiole (portion subjacent to the leaf), leaf (mid-vein area), stem (subjacent to the petiole) and roots (only from plantlets or greenhouse-grown plants) were cut into small sections (0.3-0.5 cm), fixed in formalin-acetic acid-alcohol, dehydrated in an ethanol series and wax embedded (Appendix II A). These were microtome-sectioned (8 µm), stained in safranin, counterstained in fast green and mounted in permount (Appendix II B). Photomicrographs were taken from prepared slides and the prints examined. The general shape, size and integrity of each organ was noted and the support tissue evaluated.

5.3 Results and Discussion:

5.3.1 MIS experiments

There was no change in the shoot fresh and dry weight, stem length, SSWC or PSDM of 'Q.E.' rose shoots shaken during the shoot multiplication stage (Table 5.1) or of plantlets shaken during the rooting stage (Table 5.2) compared with undisturbed control shoots or plantlets. These results suggest that shaking stress did not affect shoot growth in culture. Growth reduction of shoots in response to shaking may primarily be determined by the extent of leaf microclimate and gas exchange alteration resulting from MIS treatment (Grace and Thompson, 1973). Shaking of cultures may not have affected the climate within the test tubes sufficiently to affect shoot growth.

Leaf and root fresh weights and root dry weights of 'Q.E.' rose plantlets shaken during the rooting stage were not altered but leaf dry weights were significantly reduced in treatments 1 and 4 compared with non-shaken control plantlets (Table 5.3). It is unclear why a reduction in leaf dry weight occurred only in those plantlets shaken at the lowest speed for the shortest amount of time and the highest speed for the longest amount of time.

Table 5.1 The effect of shaking 'Q.E.' rose shoots for 4 weeks during the shoot multiplication stage at 150 or 200 rpm for 15 or 30 min on shoot fresh and dry weight, stem length, specific shoot water content (SSWC) and percent shoot dry matter (PSDM).

Treatment	Shoot		Stem		
	Fresh Wt (g)	Dry Wt (g)	length (cm)	SSWC (g H_2O g dry wt^{-1})	PSDM (%)
0 Control	0.59ab[z]	0.080a	2.32a	6.432a	13.61a
1 150 rpm X 15′	0.61a	0.084a	2.80a	6.336a	13.76a
2 150 rpm X 30′	0.60a	0.081a	2.41a	6.414a	13.57a
3 200 rpm X 15′	0.46b	0.071a	2.32a	5.390a	15.94a
4 200 rpm X 30′	0.55b	0.080a	3.15a	5.900a	14 59a

z Mean separation of treatments (Duncan's multiple range test at the 5 % level)

Table 5.2 The effect of shaking 'Q.E.' rose plantlets for 2 weeks during the rooting stage at 150 or 200 rpm for 15 or 30 min on shoot fresh and dry weight, stem length, specific shoot water content (SSWC) and percent shoot dry matter (PSDM).

Treatment	Shoot		Stem		
	Fresh Wt (g)	Dry Wt (g)	length (cm)	SSWC (g H_2O g dry wt^{-1})	PSDM (%)
0 Control	0.125a[z]	0.020a	2.50ab	4.42ab	19.08ab
1 150 rpm X 15′	0.120a	0.019a	2.60ab	5.08ab	16.72ab
2 150 rpm X 30′	0.146a	0.020a	2.73ab	4.79ab	17.31ab
3 200 rpm X 15′	0.125a	0.021a	2.26b	3.97b	21.21a
4 200 rpm X 30′	0.133a	0.019a	2.84a	5.29a	16.41b

z Mean separation of treatments (Duncan's multiple range test at the 5 % level)

Table 5.3 The effect of shaking 'Q.E.' rose plantlets for 2 weeks during the rooting stage at 150 or 200 rpm for 15 or 30 min on leaf and root fresh and dry weight.

Treatment	Leaf		Root	
	Fresh Wt (g)	Dry Wt (g)	Fresh Wt (g)	Dry Wt (g)
0 Control	0.16a[z]	0.034a	0.075a	0.006a
1 150 rpm X 15'	0.10a	0.020b	0.063a	0.006a
2 150 rpm X 30'	0.12a	0.027ab	0.046a	0.005a
3 200 rpm X 15'	0.12a	0.029ab	0.048a	0.006a
4 200 rpm X 30'	0.09a	0.017b	0.053a	0.004a

z Mean separation of treatments (Duncan's multiple range test at the 5 % level)

The SLWC and PLDM of 'Q.E.' rose plantlets were not affected by shaking whereas the SRWC and PRDM were significantly reduced and increased respectively in 'Q.E.' rose plantlets after treatment 3 was applied during the rooting stage (Table 5.4). Mechanical stress treatment (thigmic or seismic stress) also had no effect on SLWC of eggplant seedlings grown in outdoor or greenhouse environments. However, SLWC of contol eggplant seedlings was significantly lower in outdoor environments compared with greenhouse environments (Latimer et al., 1986). The variation in plant growth responses obtained between environments, as well as between mechanical stress treatments within environments, reinforces the concept that environmental conditions control or modify the extent and direction of plant response to controlled mechanical stress treatment (Latimer and Mitchell, 1988; Jaffe, 1976b).

Table 5.4 The effect of shaking 'Q.E.' rose plantlets fo. 2 weeks during the rooting stage at 150 or 200 rpm for 15 or 30 min on specific leaf (SLWC) and root (SRWC) water content and percent leaf (PLDM) and root (PRDM) dry matter.

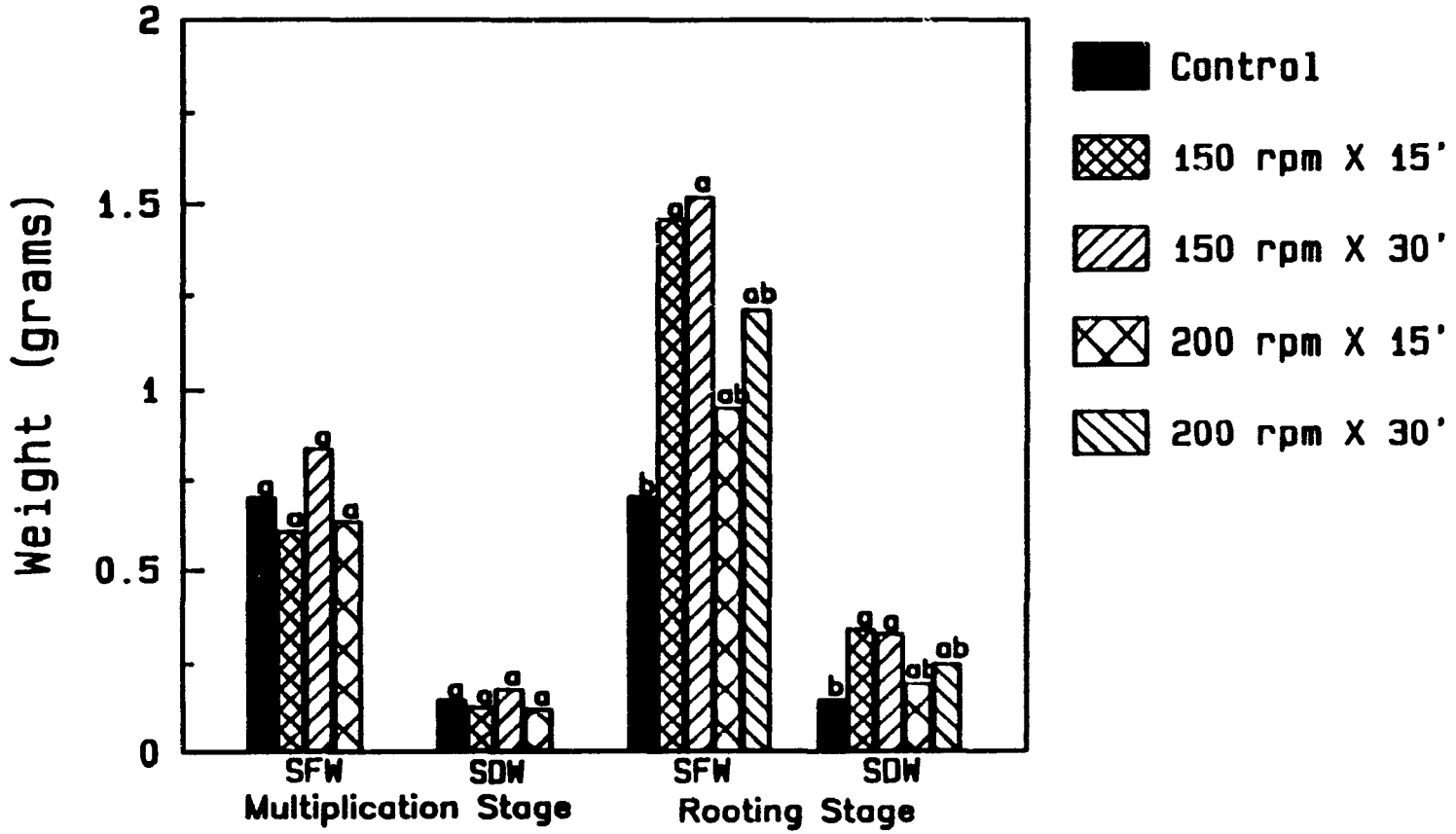
Treatment	Leaf		Root	
	SLWC (g H_2O g dry wt^{-1})	PLDM (%)	SRWC (g H_2O g dry wt^{-1})	PRDM (%)
0 Control	3.56a[z]	21.90ab	10.83a	9.20b
1 150 rpm X 15'	4.29a	19.34b	9.55ab	9.06b
2 150 rpm X 30'	3.43a	22.81ab	9.61ab	10.83b
3 200 rpm X 15'	3.19a	24.01a	5.99b	16.30a
4 200 rpm X 30'	4.54a	19.14a	9.97ab	9.66b

z Mean separation of treatments (Duncan's multiple range test at the 5 % level)

Of eighteen plantlets per treatment none survived after 2 weeks of acclimatization in a growth chamber with a light intensity of 55.95 $\mu mol.m^{-2}.s^{-1}$ (first two trials). In the third trial, when the light intensity in the growth chamber was increased from 55.95 to 124.74 $\mu mol.m^{-2}.s^{-1}$, 13 of 18 'Q.E.' rose plantlets survived. All 'Q.E.' rose plantlets derived from treatment 4 applied during the shoot multiplication stage died during the 5 weeks of acclimatization. The cause for this death is unknown.

After the acclimatization period, shoot fresh and dry weights of 'Q.E.' plants derived from treatments 1 and 2 applied during the rooting stage were significantly lower than the controls but were not altered in plants that were shaken during the shoot multiplication stage (Figure 5.1).

Figure 5.1 The effect of shaking during shoot multiplication and rooting stages on shoot fresh and dry weight of 'Q.E.' rose plants after 5 weeks of acclimatization. Different upper case letters indicate mean separation of treatments (Duncan's multiple range test, 5 % level).


Weight (grams)
2
1.5
1
0.5
0
SFW
SDW
SFW
SDW
Multiplication Stage
Rooting Stage
Control
150 rpm X 15'
150 rpm X 30'
200 rpm X 15'
200 rpm X 30'

These results compare with reports on shoot fresh and dry weight reduction in many MIS-treated herbaceous, greenhouse-grown plant species (Akers and Mitchell, 1984; Biddington and Dearman, 1985a; Latimer et al., 1986).

There was no change in root fresh weight of 'Q.E.' plants, shaken at either the shoot multiplication or root initiation stages, after acclimatization. Root dry weights of 'Q.E.' plants derived from treatment 1 applied during the rooting stage were significantly greater than the undisturbed controls after acclimatization (Figure 5.2).

After acclimatization, the root:shoot dry weight ratio was not altered in plants that were shaken during the shoot multiplication stage but was significantly less in 'Q.E.' plants derived from treatment 2 applied during the rooting stage compared with non-shaken plants (Figure 5.3). It is likely that the reductions in root and shoot dry weights of 'Q.E.' plants derived from treatment 1 applied during the rooting stage were proportional resulting in no significant change in the ratio of these two parameters.

Figure 5.2 The effect of shaking during shoot multiplication and rooting stages on root fresh and dry weight of 'Q.E.' rose plants after 5 weeks of acclimatization. Different upper case letters indicate mean separation of treatments (Duncan's multiple range test, 5 % level).

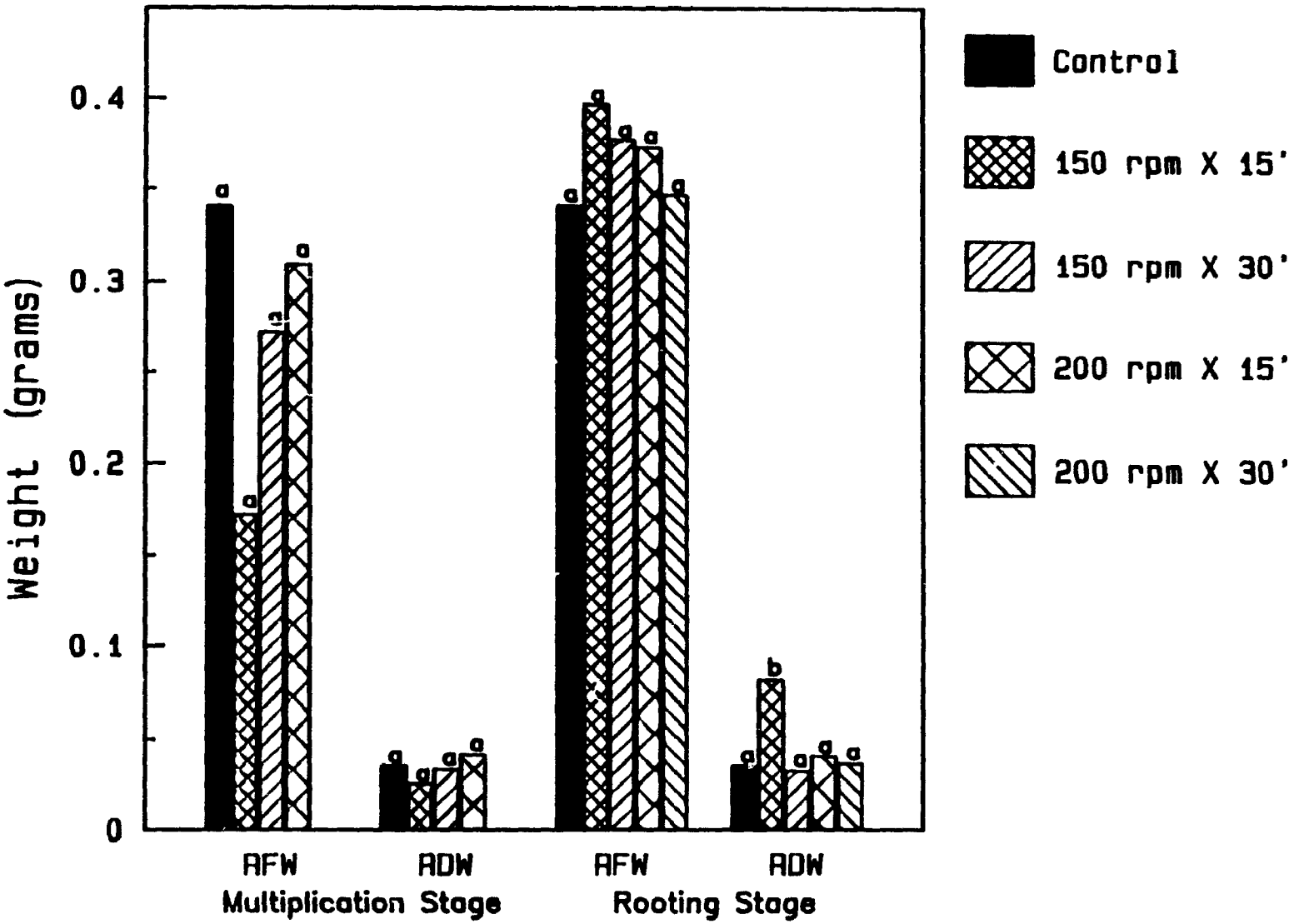
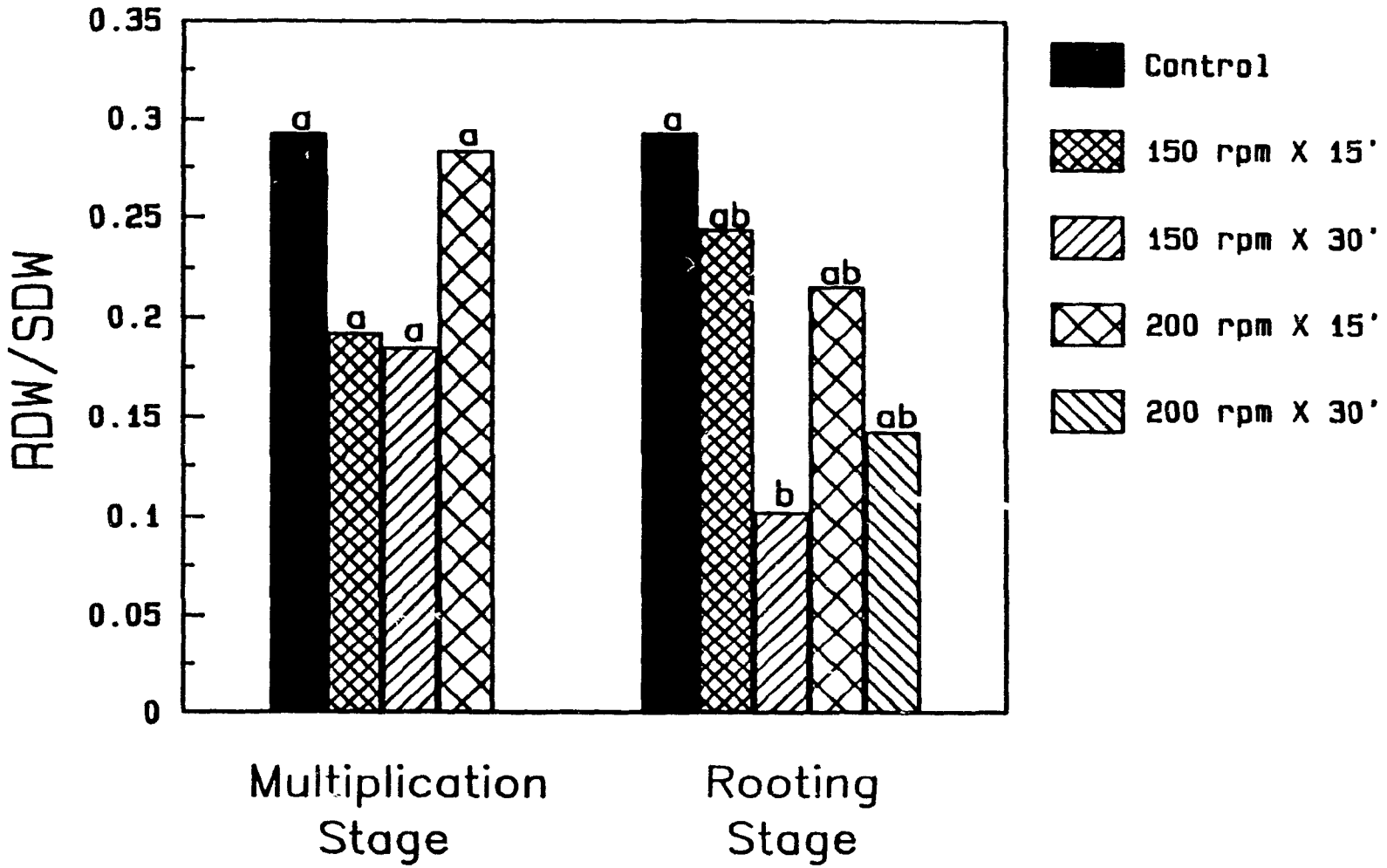

Weight (grams)
0.4
0.3
0.2
0.1
0
a
a
a
a
a
a
a
a
a
a
a
b
a
a
a
RFW
RDW
RFW
RDW
Multiplication Stage
Rooting Stage
Control
150 rpm X 15'
150 rpm X 30'
200 rpm X 15'
200 rpm X 30'

Figure 5.3 The effect of shaking during shoot multiplication and rooting stages on root (RDW): shoot (SDW) dry weight ratio of 'Q.E.' rose plants after 5 weeks of acclimatization. Different upper case letters indicate mean separation of treatments (Duncan's multiple range test, 5 % level).


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
RDW/SDW
a
a
a
a
a
ab
b
ab
ab
Control
150 rpm X 15'
150 rpm X 30'
200 rpm X 15'
200 rpm X 30'
Multiplication Stage
Rooting Stage

The relative change in root dry weight with respect to shoot dry weight in MIS-treated greenhouse-grown plants differed among plant species. Root:shoot dry weight ratio was increased in brushed lettuce, decreased in celery and unaltered in cauliflower seedlings (Biddington and dearman, 1985a).

At the end of the acclimatization period, stem and root elongation of 'Q.E.' plants that were shaken during the shoot multiplication stage were similar to unshaken plants (Table 5.5). However, there was a significant increase in root and stem elongation in plants derived from treatment 1 applied during the rooting stage, relative to undisturbed control plants (Table 5.5). It is unclear why these results contradict previous reports on many greenhouse-grown plant species whereby mechanical stimulation inhibited stem (Heuchert and Mitchell, 1983; Hammer et al., 1974; Akers and Mitchell, 1984) and root (Biddington and Dearman, 1985a) elongation.

Table 5.5 The effect of shaking during the shoot multiplication or rooting stages on stem and root elongation of 'Q.E.' rose plants after 5 weeks of acclimatization.

Treatment	Stem length (cm)	Root length (cm)
Multiplication Stage		
0 Control	5.322a[z]	5.867a
1 150 rpm X 15'	5.186a	4.714a
2 150 rpm X 30'	7.680a	5.000a
3 200 rpm X 15'	6.530a	5.150a
4 200 rpm X 30'	-	-
Rooting Stage		
0 Control	5.322b	5.867b
1 150 rpm X 15'	10.625a	9.475a
2 150 rpm X 30'	6.833ab	5.433b
3 200 rpm X 15'	5.500b	6.990ab
4 200 rpm X 30'	8.833ab	7.011ab

z Mean separation of treatments (Duncan's multiple range test at the 5 % level)

The SSWC, PSDM and SRWC of 'Q.E.' plants derived from treatments applied at either the shoot multiplication or root initiation stages did not change compared with non-shaken control plants (Table 5.6). The percent root dry matter increased significantly in 'Q.E.'plants derived from treatment 3 applied during the shoot multiplication stage and treatment 1 applied during the rooting stage, compared with non-shaken plants (Table 5.6).

Table 5.6 The effect of shaking during the shoot multiplication and rooting stages on specific shoot (SSWC) and root (SRWC) water content and percent shoot (PSDM) and root (PRDM) dry matter of 'Q.E.' rose plants after 5 weeks of acclimatization.

Treatment	Shoot		Root	
	SSWC (g H_2O g dry wt^{-1})	PSDM (%)	SRWC (g H_2O g dry wt^{-1})	PRDM (%)
Multiplication Stage				
0 Control	4.063a[z]	20.174a	7.480a	12.712b
1 150 rpm X 15'	3.797a	20.973a	5.101a	16.733ab
2 150 rpm X 30'	3.791a	21.036a	5.822a	16.558ab
3 200 rpm X 15'	4.490a	18.336a	4.783a	22.312a
4 200 rpm X 30'	-	-	-	-
Rooting Stage				
0 Control	4.063a	20.744ab	7.482a	2.712b
1 150 rpm X 15'	4.817a	28.887a	8.771a	34.118a
2 150 rpm X 30'	4.356a	19.677b	9.826a	9.384b
3 200 rpm X 15'	3.907a	21.042ab	8.086a	11.681b
4 200 rpm X 30'	3.835a	20.985ab	8.825a	10.490b

z Mean separation of treatments (Duncan's multiple range test at the 5 % level)

These results indicate no particular beneficial or harmful effects of shaking stress applied during the shoot multiplication or rooting stages on micropropagated 'Q.E.' rose shoots or plantlets. Shaking stress applied during the shoot multiplication or rooting stage did not interfere with normal in vitro shoot or root development and growth. Changes in leaf dry weight, SRWC and PRDM of 'Q.E.' plantlets after shaking for 2 weeks during the rooting stage were inconsistent. It is not clear why morphological changes that were detected after acclimatization of 'Q.E.' plantlets occurred only in plantlets derived from the

lowest-speed shaking treatments (treatments 1 and 2) applied during the rooting stage. It is possible that these changes that occurred after acclimatization were not caused by the shaking treatment applied previously in culture since once the mechanical stimulus is removed, plants resumed normal growth (Jaffe, 1973). MIS treatment has not been previously applied *in vitro*, and few reports exist on MIS treatment of woody greenhouse-grown species, thus explanations are not available from the literature.

5.3.2 Internal anatomy

The internal anatomy of mechanically-perturbed 'Q.E.' rose shoots and plantlets was similar to undisturbed controls. Collenchyma and sclerenchyma mechanical tissues were not apparently increased as a result of shaking in any of the organs observed compared with non-shaken control plants. Cross sections of mechanically-stressed petioles derived from shaking treatments applied during the shoot multiplication stage and cross sections of leaves derived from treatments 3 and 4 applied at the shoot multiplication or the rooting stages were chosen to illustrate similar support tissue formation in both shaken and non-shaken 'Q.E.' plants. In addition, support tissue formation was compared between non-shaken, micropropagated plantlets and

greenhouse-grown 'Q.E.' plants.

Petioles of mechanically-stressed (treatments 1, 2, 4 applied during the shoot multiplication stage) or non-stressed tissue cultured shoots of 'Q.E.' rose had similar anatomy (Figure 5.4 A, B, C, D). Petioles of *in vitro* propagated shoots (shaken or non-shaken) had a single layer of collenchyma cells just beneath the epidermis and two to three layers of sclerenchyma cells in the phloem. This is in contrast to an increase in collenchyma cell formation in celery petioles exposed to wind (Venning, 1949). Petioles of greenhouse-grown control plants had one or two more layers of both collenchyma and sclerenchyma cells (Figure 5.4 E).

Collenchyma and sclerenchyma tissues in 'Q.E.' leaves derived from shoots or plantlets that were shaken during the shoot multiplication (treatment 3 and 4; Figure 5.5 C and D) and root initiation stages (treatment 3 and 4; Figure 5.5 E and F) were similar to the undisturbed control plants (Figure 5.5 A). This is in contrast to an increase in leaf sclerenchyma in corn plants exposed to wind (Whitehead and Luti, 1961).

Figure 5.4 Photomicrographs of cross sections of petioles of control tissue-cultured (A), treatments 1 (B), 2 (C) and 4 (D) applied during the shoot multiplication stage *in vitro* and control greenhouse-grown (E) 'Q.E.' rose (Epidermis, e; Phloem, ph; Sclerenchyma, s; Collenchyma, c).
Bar = 200 µm

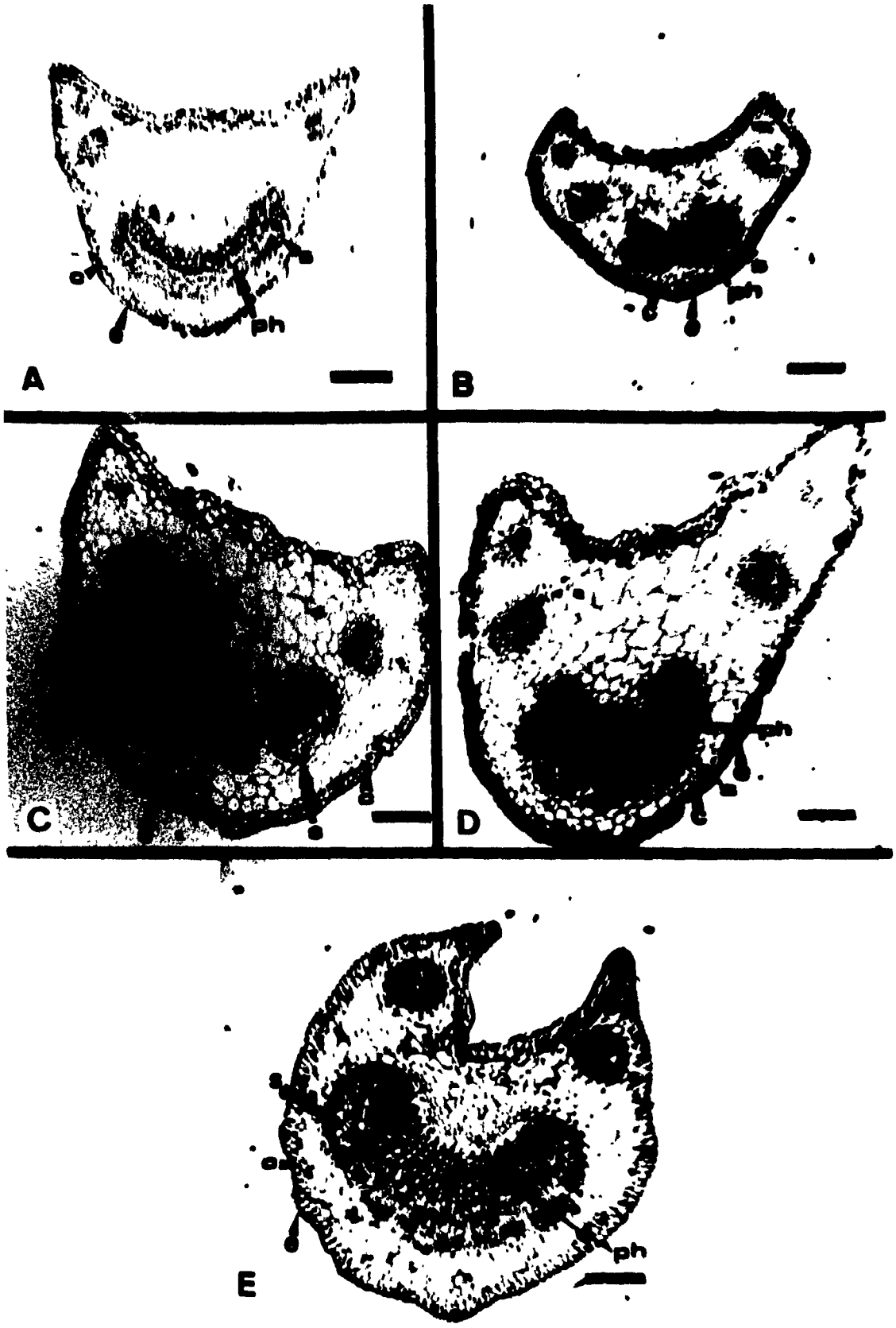
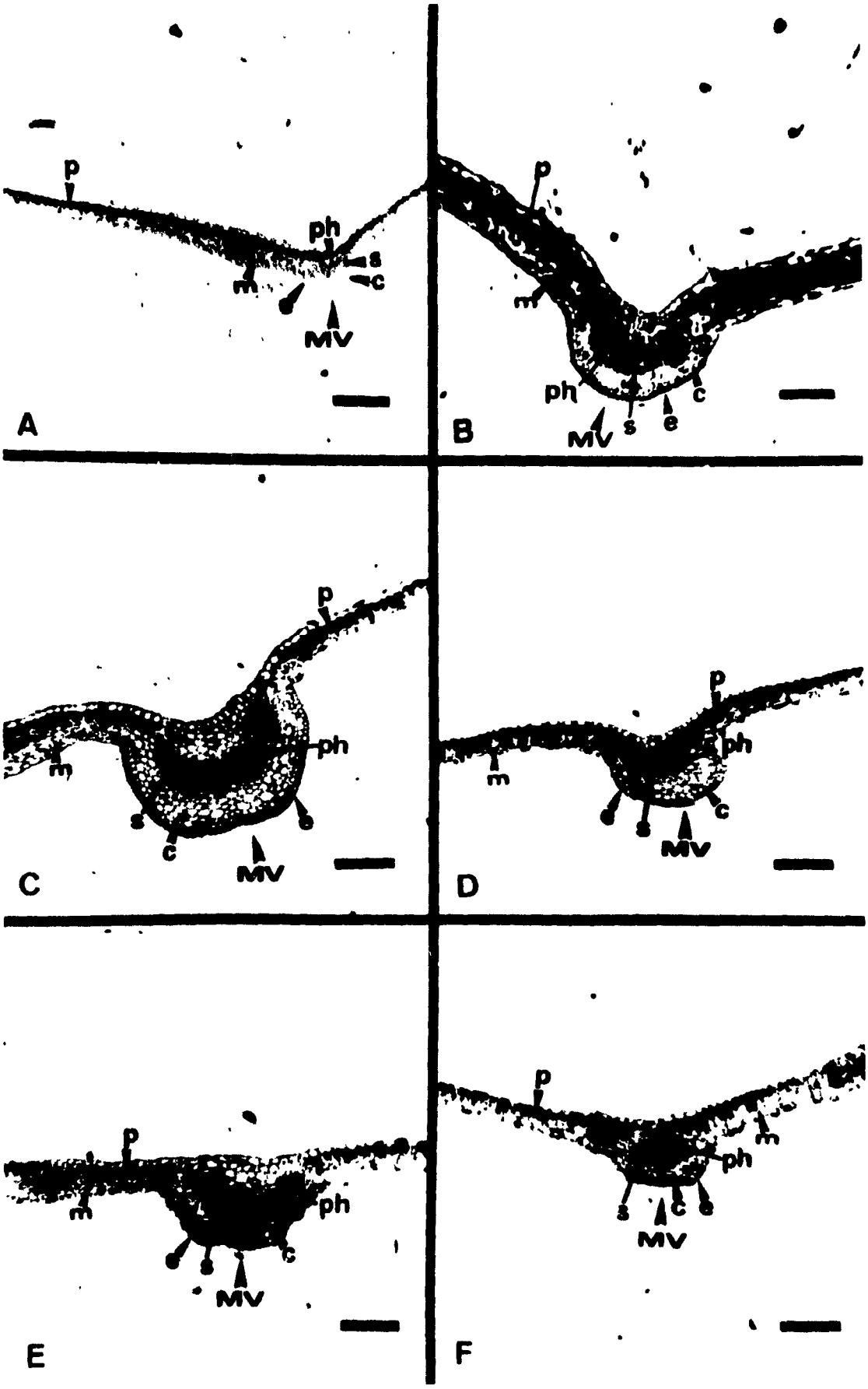

A
ph
B
ph
C
D
ph
E
ph

Figure 5.5 Photomicrographs of cross sections of leaves of control tissue-cultured (A), control greenhouse-grown (B), treatments 3 (C) and 4 (D) applied during the shoot multiplication stage and treatments 3 (E), 4 (F) applied during the rooting stage *in vitro* of 'Q.E.' rose (Epidermis, e; Phloem, ph; Sclerenchyma, s; Collenchyma,c; Mid Vein, MV; Palisade, p; Mesophyll, m). Bar = 200 μm


p
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B
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s
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C
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m
ph
c
MV
D
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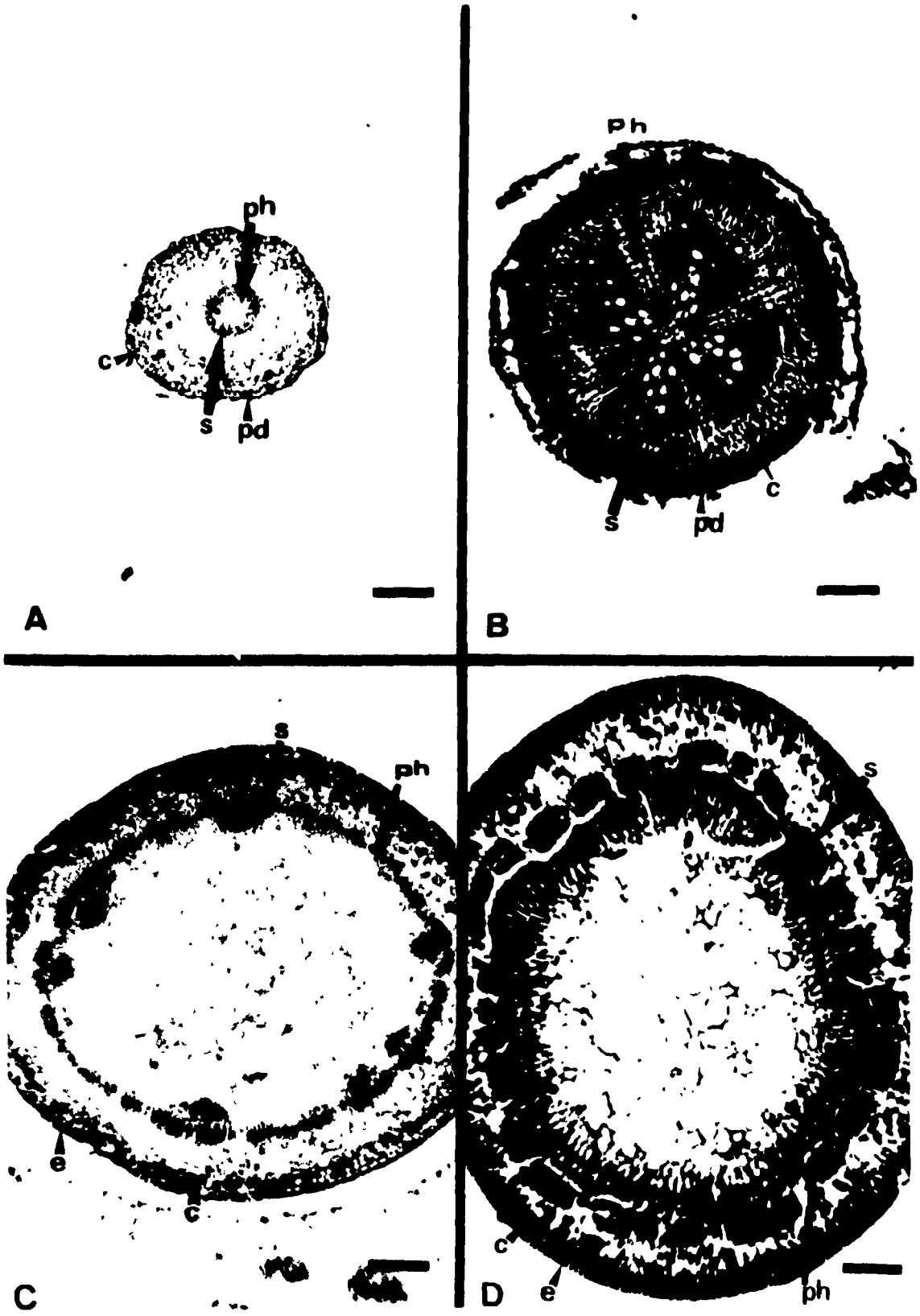
m
ph
MV
E
p
m
ph
s
c
e
MV
F

Tissue-cultured plantlet leaves had one layer of palisade cells with two to three layers of spongy mesophyll cells that were loosely arranged, with large intercellular spaces between them. In the mid vein area, a distinct layer of collenchyma showed beneath the epidermis and one-two layers of sclerenchyma surrounded the phloem (Figure 5.5 A). In contrast, mature greenhouse-grown leaves had two to three distinct layers of palisade cells with two to three rows of densely packed spongy mesophyll cells. The leaf mid-vein had two to three layers of collenchyma beneath the epidermis and two to three layers of sclerenchyma cells in the phloem (Figure 5.5 B).

Shaking 'Q.E.' shoots or plantlets did not affect support tissue formation in either roots or stems . Roots of control (or shaken) plantlets were white in color, had little periderm and two to three layers of collenchyma beneath the epidermis. A distinct layer of sclerenchyma cells capped the phloem. In contrast, roots of greenhouse-grown 'Q.E.'rose were brown in color, with a multilayered periderm, three to four layers of collenchyma cells beneath the epidermis and two to three layers of sclerenchyma cells in the phloem (Figure 5.6 A and B).

Figure 5.6 Photomicrographs of cross sections of roots (A and B) and stems(C and D) of control tissue-cultured (left) and greenhouse-grown (right) 'Q.E.' rose (Periderm, pd; Epidermis, e; Phloem, ph; Sclerenchyma, s; Collenchyma, c)

Bar = 200 μm


ph
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A
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C
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D

Stems of control (or shaken) micropropagated plantlets had two to three layers of collenchyma cells in the cortex compared with four to five layers of these cells in greenhouse-grown plants. Sclerenchyma cells were thicker-walled and denser in greenhouse-grown plants compared with tissue-cultured plantlets (Figure 5.4 C and D).

It is clear from these anatomical observations that mechanical stress applied as shaking stress, in vitro, did not promote mechanical tissue formation as was reported for in vivo celery (Venning, 1949) or corn (Whitehead and Luti, 1961) plants exposed to wind. This underlines the fact that shaking stress treatment was ineffective for roses when applied in vitro.

Tissue cultured plantlets seem to form less strengthening tissues compared with greenhouse-grown 'Q.E.' plants. Such differences in anatomical features in roots, stems and petioles were previously reported between tissue-cultured and greenhouse-grown red raspberry and were attributed to the nature of the tissue culture environment (Donnelly et al., 1985). Leaves of control tissue-cultured red raspberry and sweetgum plantlets had also less support tissue than greenhouse-grown plants (Donnelly and Vidaver, 1984a; Wetzstein and Sommer, 1982). Support tissues in these plants may have been suppressed or inhibited by the mechanical stress-free in vitro cultural conditions compared with greenhouse or field environmental conditions

(Donnelly et al., 1985).

5.4 Summary and Conclusion:

Shaking 'Q.E.' rose shoots or plantlets, in culture, did not affect shoot parameters such as shoot fresh and dry weight, stem length, SSWC and PSDM. However, when shaking was done at the rooting stage at 150 rpm for 15 min daily for 2 weeks leaf dry weight of 'Q.E.' plantlets were significantly reduced prior to acclimatization. Also, shaking 'Q.E.' plantlets at the rooting stage at 200 rpm for 15 min daily significantly reduced the SRWC but increased the PRDM accumulation, prior to acclimatization. There was a significant reduction in shoot fresh and dry weights of acclimatized 'Q.E.' plants that were shaken during the rooting stage at 150 rpm for 15 or 30 min. The root dry weight and the PRDM were reduced and stem and root elongation were increased after acclimatization of 'Q.E.' plants that were shaken during the rooting stage at 150 rpm for 15 min. Root:shoot dry weight ratio was significantly reduced in acclimatized 'Q.E.' plantlets shaken during the rooting stage at 150 rpm for 30 min daily for 2 weeks.

All 'Q.E.' plantlets, except those derived from treatment 4 applied during the shoot multiplication stage, survived acclimatization. Morphological changes detected

after acclimatization could be due to the favorable environmental conditions in the growth chambers that masked the effects of mechanical stress since once the mechanical stimulus is removed, plants resume normal growth after several days (Jaffe, 1973). Also, since shoot parameters were not obviously affected by the shaking stress applied at either stage and only leaf dry weights, SRWC and PRDM changed following shaking for 2 weeks during the rooting stage, it is likely that shaking treatments in vitro are ineffective. Anatomical observations support this fact since mechanical tissue formation was similar in stressed and unstressed tissue-cultured 'Q.E.' shoots or plantlets.

There are several possible reasons why MIS treatments may have been ineffective in inhibiting growth and improving strength and hardiness of in vitro 'Q.E. plantlets. First, it is possible that the shaking speeds and durations selected, although useful for herbaceous species, were insufficient for a woody species like rose. Second, the efficacy of the treatments may have been hindered by the saturated environmental conditions inside the culture tubes. Shaking action usually alters the leaf microclimate and gas exchange (Grace and Thompson, 1973) resulting in an increased transpiration rate followed by a reduced water potential causing growth inhibition. Shaking is likely to have disturbed the air inside the tubes. However, the relatively high humidity level in the culture

tubes depresses the transpiration rate. In addition, there is a relatively poor gas exchange in the closed tubes. Third, temperature and light level are among the environmental variables known to modify the responsiveness of plants to mecnanical stress. For example, bean plants had characteristic minimum and maximum optimum temperatures for growth inhibition in reponse to thigmic stress (Jaffe, 1976a), as did chrysanthemum for seismic stress (Beyl and Mitchell, 1977b). Also a progressive decrease in sensitivity to shaking stress with increasing light intensity was reported for tomato (Heuchert and Mitchell, 1983) and soybean (Pappas and Mitchell, 1985). Temperatures and light intensities in the culture room may not have been ideal to maximize the effects of shaking stress when applied during the shoot multiplication and rooting stages in vitro. Fourth, the relative locations of sources and sinks determine growth and support tissue deposition. Any growing, actively metabolizing tissue such as developing young leaves, roots and stems act as sinks for photosynthates. When active growth terminates, cell wall deposition continues. Photosynthetically active leaves typically constitute the sources and the assimilates move from the sources to the sinks (Salisbury and Ross, 1978). In the tissue culture environment, exogenously supplied sugars and nutrients are presumably easily translocated to newly developing tissues but less strengthening tissue is

deposited. Nutritionally, the medium may limit synthetic processes leading to additional cell wall deposition.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Preliminary tissue culture experiments allowed the establishment of a micropropagation system for 'Q.E.' and 'D.K.' roses. Nodal stem segments of both cultivars produced seven-ten new shoots per month on a MS basal medium supplemented with low rates of BAP (0.1-1.0 mg/liter) and NAA (0-0.5 mg/liter). Rooting of 'Q.E.' but not 'D.K.' rose was successfully achieved on a MS basal medium amended with 1 mg/liter IBA. Future research studies on roses should envisage the following: (1) testing types and concentrations of growth regulators at different stages of micropropagation to improve production capacity and quality of the specific variety being investigated, (2) optimizing environmental conditions such as light quality and intensity, temperature and relative humidity at all micropropagation stages especially to improve *ex vitro* transplantation and survival of the rose plantlets under study and (3) testing production quality and quantity of *in vitro* versus *in vivo* propagated roses. However, such studies should be specific to each variety of rose and may not necessarily be extrapolated within species.

Automated shaking stress on conventionally-propagated 'Q.E.' rose cuttings at 200 rpm for 30 min daily improved rooting by increasing the root dry weight, root:shoot dry weight and root length. Shaking 'D.K.' rose cuttings at 200 rpm for 15 min increased shoot fresh and dry weight as well as root dry weight and root length. However, the experiments were discontinued due to disease and further evidence is required to assess effects of mechanical stress on early establishment of rose cuttings. It is recommended that future experimental work related to the effects of mechanical stress on rose cuttings should consider the following : (1) using disease-free stock plants and conducting work in highly controlled environments (Akers and Mitchell, 1984) and (2) using different varieties of the same species or of different species to detect whether MIS responses are species or variety-related. In addition, future research work on the effects of MIS on roses could include: (1) using different types of mechanical stimuli such as a "thigmostimulator" (Beyl and Mitchell, 1977a) or "mechanical fingers" (Jaffe, 1980) and (2) testing a wide range of stimulus intensities and durations to optimize the application. Once morphological changes are detected, work on the mechanisms involved in the rose plant responses to mechanical stimulus and this should include: (1) testing the levels of endogenous hormones following the mechanical stress application, (2)

testing the electrical resistance of rose plants under stress, (3) studying stomatal conductance and other physiological processes such as photosynthetic activity, transpiration and respiration rates and (4) evaluating mechanical tissue deposition and the mechanisms involved with this process. However, this type of research could not necessarily be extrapolated from one species to another since different plants respond differently to MIS.

Automated shaking stress at the shoot multiplication and rooting stages of micropropagated 'Q.E.' rose did not alter shoot growth parameters. Shaking during the rooting stage at 150 rpm for 15 min daily for 2 weeks reduced leaf dry weight of 'Q.E.' plantlets prior to transfer to soil. Also, shaking 'Q.E.' plantlets at the same stage at 200 rpm for 15 min daily reduced SRWC and increased the PRDM, prior to acclimatization. After acclimatization, shoot fresh and dry weights were reduced in 'Q.E.' plants exposed to shaking treatments of 150 rpm for 15 and 30 min during the rooting stage. Root dry weight and PRDM were reduced and stem and root length were increased in acclimatized 'Q.E.' plants shaken at 150 rpm for 15 min during the rooting stage. After acclimatization, root:shoot dry weight ratio was significantly reduced in 'Q.E.' plants shaken during the rooting stage at 150 rpm for 30 min.

The internal anatomy of mechanically-perturbed in

vitro 'Q.E.' shoots and plantlets remained unchanged, without any obvious additional mechanical tissue formation. Although some morphological changes were detected prior to or after acclimatization in micropropagated 'Q.E.' rose mechanically-stressed during the rooting stage, MIS seemed not to have an effect on shoot and plantlet growth. *Ex vitro* survival was successful for both stressed and unstressed 'Q.E.' plantlets. The long term effects of MIS on *ex vitro* plantlet survival and hardiness require further assessment. Prior to conducting future studies, shaking stress or any other type of MIS must be proven effective *in vitro*. For this, a species, extensively reported in the literature as sensitive to MIS *in vivo* should be tested. If MIS treatment was effective *in vitro*, similar experiments as the ones presented in this manuscript could be conducted to meet similar objectives.

CHAPTER VII

CLAIMS OF ORIGINALITY

The following points describe original contributions to knowledge:

1. The specific requirements for the successful micropropagation of 'Q.E.' and 'D.K.' roses were established. A seven-ten fold multiplication rate per month was achieved from nodal stem explants on MS basal medium supplemented with low rates of BAP (0.1-1.0 mg/liter) and NAA (0-0.5 mg/liter). Root initiation of 'Q.E.' rose was achieved on MS basal medium supplemented with 1 mg/liter IBA.

2. It was determined that automated shaking stress improved rooting of conventionally-propagated 'Q.E.' and 'D.K.' roses but further evidence of its effects on early establishment still need to be assessed.

3. It was determined that automated shaking stress did not result in any significant change in plant size, growth rate or dry weight acumulation when applied to micropropagated

'Q.E.' roses during the shoot multiplication stage. Some morphological changes were detected when the stress was applied during the rooting stage. However, mechanical stress in culture did not show any obvious improvement on ex vitro survival and hardiness of rose plantlets since both shaken and non-shaken shoots and plantlets successfully survived the acclimatization stage.

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APPENDIX I

MURASHIGE AND SKOOG BASAL MEDIUM (1962)

COMPOSITION

Stock Solution (SS)	Constituents	Concentration in SS (g/l)	Volume of SS in medium (ml)	Concentr. in medium (mg/l)
A	NH_4NO_3	82.50	20	1650.0
B	KNO_3	95.00	20	1900.0
C	H_3BO_3	1.24	5	6.2
	KH_2PO_4	34.00		170.0
	KI	0.166		0.83
	$Na_2MoO_4.2H_2O$	0.05		0.25
	$CoCl_2.6H_2O$	0.005		0.02
D	$CaCl_2.2H_2O$	88.00	5	440.0
E	$MgSO_4.7H_2O$	74.00	5	370.0
	$MnSO_4.4H_2O$	3.45		22.3
	$ZnSO_4.7H_2O$	1.72		8.6
	$CuSO_4.5H_2O$	0.005		0.02
F	Na_2.EDTA	7.45	5	37.35
	$FeSO_4.7H_2O$	5.57		27.85
G	Thiamine.HCl	0.20	5	1.0*
	Nicotinic acid	0.10		0.5
	Pyridoxine HCl	0.10		0.5
	Glycine	0.40		2.0

* The MS concentration of thiamine.HCl was raised to 1.0 mg/liter in these experiments as suggested by Linsmair and Skoog (1965).

One liter of MS basal medium contains the assigned volumes (5 or 20 ml) of the stock solutions, sucrose (30 g/l), myo-inositol (100 mg/l) dissolved in double distilled water and an appropriate concentration of growth regulators.

APPENDIX II

ANATOMICAL MICROTECHNIQUE

A. WAX EMBEDDING:

1.	Fixation	: Ethanol (70 %):Formalin:Acetic Acid (90:5:5; 3 days)
2.	Dehydration:	70 % Ethanol (1 day; 2 hr X 3) 70 % " (15 min) 85 % " (15 min X 2) 95 % " " 100 % " (15 min X 4)
3.	Organic Dissolution	: 50 % Ethanol:50 % Xylene (15 min) 100 % Xylene (15 min X 4)
4.	Wax Embedding	: 50 % Xylene:50 % Wax (30 min at 55°C) 100 % Wax (30 min X 3)

B. STAINING:

1.	Wax Dissolution	: 100 % Xylene (15 min X 4)
2.	Hydration	: 100 % Ethanol (10 min X 2) 95 % " (10 min) 70 % " " 50 % " " 25 % " " H_2O "
3.	Staining	: 1 % Safranin (1/2 hr-1 hr)
4.	Washing	: 3 rinses in water
5.	Counterstaining:	0.5 % Fast Green (1-2 sec)
6.	Clearing	: Clove oil (1-5 min X 2) 100 % Xylene (" X ")
7.	Mounting	: Permount (2-3 drops)

APPENDIX III

GREENHOUSE RESEARCH EXPERIMENTS

GENERAL LINEAR MODELS PROCEDURE

A. 'QUEEN ELIZABETH' ROSE:

Dependent Variable: Change in Fresh Weight (FW=FW2-FW1)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.3460	0.0869	2.77	0.0327
Error	78	2.4327	0.0312		
Corrected Total	82	2.7787			

R-Square	C.V.	Root MSE	FW Mean
0.124525	63.92530	0.176603	0.2762650

Dependent Variable: Change in Petiole Diameter (PD=PD2-PD1)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0001	0.00004	0.16	0.9562
Error	78	0.0224	0.00028		
Corrected Total	82	0.0226			

R-Square	C.V.	Root MSE	PD Mean
0.008319	89.61785	0.016952	0.0189156

Dependent Variable: Change in Stem Diameter (SD=SD2-SD1)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0082	0.0020	2.25	0.0708
Error	78	0.0710	0.0009		
Corrected Total	82	0.0792			

R-Square	C.V.	Root MSE	SD Mean
0.103615	119.8650	0.030183	0.0251807

Dependent Variable: Root Length (RL)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	7.7664	1.9416	1.76	0.1461
Error	78	186.2004	1.1051		
Corrected Total	82	93.9669			

R-Square	C.V.	Root MSE	RL Mean
0.082651	39.83112	1.051254	2.6392771

Dependent Variable: Root Number (RN)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	19.6199	4.9049	0.57	0.6850
Error	78	670.8865	8.6011		
Corrected Total	82	690.5060			

R-Square	C.V.	Root MSE	RN Mean
0.028414	53.85385	2.932764	5.4457831

Dependent Variable: Square Root of Root Number (ROOT)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1.1403	0.2850	0.67	0.6147
Error	78	33.1876	0.4254		
Corrected Total	82	34.3279			

R-Square	C.V.	Root MSE	ROOT Mean
0.033218	29.07784	0.652290	2.2432551

Dependent Variable: Shoot Fresh Weight (SFW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.5593	0.1398	5.34	0.0063
Error	16	0.4189	0.02618339		
Corrected Total	20	0.9783			

R-Square	C.V.	Root MSE	SFW Mean
0.571784	24.32404	0.161813	0.665238

Dependent Variable: Shoot Dry Weight (SDW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0333	0.0083	3.78	0.0239
Error	16	0.0353	0.0022		
Corrected Total	20	0.0687			

R-Square	C.V.	Root MSE	SDW Mean
0.48578	32.25041	0.046993	0.145714

Dependent Variable: Root Fresh Weight (RFW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0974	0.0243	3.39	0.0344
Error	16	0.1150	0.0071		
Corrected Total	20	0.2124			

R-Square	C.V.	Root MSE	RFW Mean
0.458636	47.47975	0.084785	0.178571

Dependent Variable: Root Dry Weight (RDW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0011	0.00029	6.69	0.0023
Error	16	0.0007	0.00004		
Corrected Total	20	0.0018			

R-Square	C.V.	Root MSE	RDW Mean
0.625861	41.99726	0.006640	0.0158095

Dependent Variable: RDW/SDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.1391	0.0347	12.61	0.0001
Error	16	0.0441	0.0027		
Corrected Total	20	0.1832			

R-Square	C.V.	Root MSE	RDW/SDW Mean
0.759123	42.57597	0.052528	0.12337447

Dependent Variable: Specific Shoot Water Content (SSWC)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	3.8117	0.9529	0.71	0.5969
Error	16	21.4751	1.3421		
Corrected Total	20	25.2868			

R-Square	C.V.	Root MSE	SSWC Mean
0.150739	30.45082	1.158531	3.8045968

Dependent Variable: Specific Root Water Content

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	4	128.8551	32.2137	2.27	0.1069
Error	16	227.0527	14.1908		
Corrected Total	20	355.9079			

R-Square	C.V.	Root MSE	SRWC Mean
0.362046	34.02803	3.767068	11.070483

Dependent Variable: Percent Shoot Dry Matter (PSDM)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	47.7643	11.9410	0.67	0.6209
Error	16	284.3282	17.7705		
Corrected Total	20	332.0926			

R-Square	C.V.	Root MSE	PSDM Mean
0.143829	19.42933	4.215509	21.696622

Dependent Variable: Percent Root Dry Matter (PSDM)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	110.9815	27.7453	3.55	0.0296
Error	16	125.1092	7.8193		
Corrected Total	20	236.0908			

R-Square	C.V.	Root MSE	PRDM Mean
0.470080	30.17266	2.796307	9.267684

B. 'DICK KOSTER' ROSE:

Dependent Variable: FW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.1748	0.0437	4.84	0.0016
Error	75	0.6777	0.0090		
Corrected Total	79	0.8526			

R-Square	C.V.	Root MSE	FW Mean
0.205074	59.41354	0.095062	0.16000000

Dependent Variable: PD

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0007	0.00019	0.76	0.5550
Error	75	0.0194	0.00025		
Corrected Total	79	0.0202			

R-Square	C.V.	Root MSE	PD Mean
0.038920	171.9061	0.016116	0.00937500

Dependent Variable: SD

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0017	0.00044	3.16	0.0185
Error	75	0.0106	0.00014		
Corrected Total	79	0.0124			

R-Square	C.V.	Root MSE	SD Mean
0.144343	146.4990	0.011903	0.00812500

Dependent Variable: RL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	38.6419	9.6604	4.53	0.0025
Error	75	160.0487	2.13398344		
Corrected Total	79	198.6907			

R-Square	C.V.	Root MSE	RL Mean
0.194483	47.78984	1.460816	3.05675000

Dependent Variable: RN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	200.0732	50.0183	3.72	0.0081
Error	75	1007.9142	13.4388		
Corrected Total	79	1207.9875			

R-Square	C.V.	Root MSE	RN Mean
0.165625	62.26590	3.665905	5.88750000

Dependent Variable: ROOT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	7.0842	1.7710	3.48	0.0116
Error	75	38.1912	0.5092		
Corrected Total	79	45.2755			

R-Square	C.V.	Root MSE	ROOT Mean
0.156471	30.93370	0.713594	2.30684980

Dependent Variable: SFW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0682	0.0170	4.14	0.0119
Error	22	0.0906	0.0041		
Corrected Total	26	0.1588			

R-Square	C.V.	Root MSE	SFW Mean
0.429475	32.94812	0.064188	0.1948148

Dependent Variable: SDW

Source	D	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0046	0.0011	4.05	0.0131
Error	22	0.0062	0.0002		
Corrected Total	26	0.0108			

R-Square	C.V.	Root MSE	SDW Mean
0.424116	42.61628	0.016889	0.0396296

Dependent Variable: RFW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0062	0.0015	2.33	0.0876
Error	22	0.0147	0.0006		
Corrected Total	26	0.0210			

R-Square	C.V.	Root MSE	RFW Mean
0.297766	56.44389	0.025922	0.04592593

Dependent Variable: RDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.00010	0.00002	2.29	0.0922
Error	22	0.00025	0.00001		
Corrected Total	26	0.00036			

R-Square	C.V.	Root MSE	RDW Mean
0.293787	85.89498	0.003404	0.00396296

Dependent Variable: RDW/SDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0063	0.0015	0.28	0.8877
Error	22	0.1241	0.0056		
Corrected Total	26	0.1304			

R-Square	C.V.	Root MSE	RDW/SDW Mean
0.048448	71.37449	0.075116	0.10524250

Dependent Variable: SSWC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	11.4365	2.8591	0.93	0.4638
Error	22	67.5250	3.0693		
Corrected Total	26	78.9616			

R-Square	C.V.	Root MSE	SSWC Mean
0.144837	39.37274	1.751948	4.44964727

Dependent Variable: SRWC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	189.7399	47.4349	0.79	0.5453
Error	22	1324.2459	60.1924		
Corrected Total	26	1513.9859			

R-Square	C.V.	Root MSE	SRWC Mean
0.125325	59.00370	7.758415	13.1490300

Dependent Variable: PSDM

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	202.2764	50.5691	1.63	0.2029
Error	22	683.6587	31.0753		
Corrected Total	26	885.9351			

R-Square	C.V.	Root MSE	PSDM Mean
0.228320	27.83564	5.574531	20.0265986

Dependent Variable: PRDM

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	158.9015	39.7253	2.34	0.0864
Error	22	372.8703	16.9486		
Corrected Total	26	531.7719			

R-Square	C.V.	Root MSE	PRDM Mean
0.298815	45.66921	4.116874	9.01455026

APPENDIX IV

GENERAL LINEAR MODELS PROCEDURE

TISSUE CULTURE RESEARCH EXPERIMENTS

A. END OF SHOOT MULTIPLICATION STAGE:

Dependent Variable: Shoot Fresh Weight (SFW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0738	0.0184	0.58	0.6810
Error	22	0.7019	0.0319		
Corrected Total	26	0.7758			

R-Square	C.V.	Root MSE	SFW Mean
0.095237	30.89540	0.178621	0.57814815

Dependent Variable: Shoot Dry Weight (SDW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0004	0.00012	0.95	0.4542
Error	22	0.0028	0.00013		
Corrected Total	26	0.0033			

R-Square	C.V.	Root MSE	SDW Mean
0.147265	14.45230	0.011455	0.07925926

Dependent Variable: Specific Shoot Water Content (SSWC)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	4.4591	1.1147	1.69	0.1879
Error	22	14.5026	0.6592		
Corrected Total	26	18.9618			

R-Square	C.V.	Root MSE	SSWC Mean
0.235164	13.39113	0.811919	6.06311127

Dependent Variable: Percent Shoot Dry Matter (PSDM)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	23.0089	5.7522	1.98	0.1331
Error	22	63.9597	2.9072		

Corrected Total	26	86.9687

R-Square	C.V.	Root MSE	PSDM Mean
0.264566	11.86644	1.705070	14.3688416

Dependent Variable: Shoot Length (SL)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	2.8923	0.7230	1.03	0.4128
Error	21	14.6788	0.6989		
Corrected Total	25	17.5711			

R-Square	C.V.	Root MSE	SL Mean
0.164606	31.82649	0.836057	2.62692308

B. END OF ROOT INITIATION STAGE:

Dependent Variable: SFW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0025	0.00064	0.29	0.8813
Error	23	0.0511	0.00222		
Corrected Total	27	0.0536			

R-Square	C.V.	Root MSE	SFW Mean
0.048043	36.16450	0.047143	0.13035714

Dependent Variable: SDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.00006	0.00001	0.50	0.7345
Error	23	0.00075	0.00003		
Corrected Total	27	0.00082			

R-Square	C.V.	Root MSE	SDW Mean
0.080310	29.67438	0.005734	0.01932143

Dependent Variable: RFW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0026	0.00067	1.21	0.3346
Error	23	0.0127	0.00055		
Corrected Total	27	0.0154			

R-Square	C.V.	Root MSE	RFW Mean
0.173555	42.04521	0.023575	0.05607143

Dependent Variable: Root Dry Weight (RDW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.00001	0.000003	0.67	0.6226
Error	23	0.00013	0.000005		
Corrected Total	27	0.00014			

R-Square	C.V.	Root MSE	RDW Mean
0.103689	41.26898	0.002402	0.00582143

Dependent Variable: RDW/SDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.02015	0.00503	1.45	0.2487
Error	23	0.07974	0.00346		
Corrected Total	27	0.09989			

R-Square	C.V.	Root MSE	RDW/SDW Mean
0.201776	42.92402	0.058882	0.13717634

Dependent Variable: Leaf Fresh Weight (LFW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.01175	0.00293	1.09	0.3835
Error	23	0.06183	0.00268		
Corrected Total	27	0.07358			

R-Square	C.V.	Root MSE	LFW Mean
0.159710	43.46693	0.051850	0.11928571

Dependent Variable: Leaf Dry Weight (LDW)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.00097	0.00024	2.24	0.0958
Error	23	0.00251	0.00010		
Corrected Total	27	0.00349			

R-Square	C.V.	Root MSE	LDW Mean
0.280563	41.21886	0.010452	0.025357

Dependent Variable: SSWC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	6.4551	1.6137	1.88	0.1486
Error	23	19.7698	0.8595		
Corrected Total	27	26.2249			

R-Square	C.V.	Root MSE	SSWC Mean
0.246145	19.57486	0.927123	4.73629477

Dependent Variable: Specific Leaf Water Content (SLWC)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	7.9779	1.9944	2.06	0.1191
Error	23	22.2557	0.9676		
Corrected Total	27	30.2336			

R-Square	C.V.	Root MSE	SLWC Mean
0.263875	25.72797	0.983687	3.82341348

Dependent Variable: PSDM

Source	DF	Sum of Square	Mean Square	F Value	Pr > F
Model	4	99.7382	24.9345	2.06	0.1189
Error	23	278.1195	12.0921		
Corrected Total	27	377.8577			

R-Square	C.V.	Root MSE	PSDM Mean
0.263957	19.28912	3.477377	18.0276634

Dependent Variable: SRWC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	77.1616	19.2904	1.66	0.1926
Error	23	266.6803	11.5947		
Corrected Total	27	343.8419			

R-Square	C.V.	Root MSE	SRWC Mean
0.224410	37.51217	3.405113	9.07735518

Dependent Variable: PRDM

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	197.5048	49.3762	2.59	0.0635
Error	23	438.4916	19.0648		
Corrected Total	27	635.9965			

R-Square	C.V.	Root MSE	PRDM Mean
0.310544	38.59209	4.366332	11.3140590

Dependent Variable: Percent Leaf Dry Matter (PLDM)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	109.6765	27.4191	3.22	0.0309
Error	23	195.9902	8.5213		
Corrected Total	27	305.6668			

R-Square	C.V.	Root MSE	PLDM Mean
0.358811	13.63365	2.919130	21.4112095

C. END OF ACCLIMATIZATION STAGE:

1. Experiment 1:

Dependent Variable: SFW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.1625	0.0541	0.35	0.7919
Error	20	3.1254	0.1562		
Corrected Total	23	3.2880			

R-Square	C.V.	Root MSE	SFW Mean
0.049431	56.87980	0.395315	0.69500000

Dependent Variable: SDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.0094	0.00314	0.37	0.7759
Error	20	0.1705	0.00852		
Corrected Total	23	0.1799			

R-Square	C.V.	Root MSE	SDW Mean
0.052507	63.75453	0.092338	0.14483333

Dependent Variable: RFW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.1160	0.0386	0.63	0.6015
Error	20	1.2195	0.0609		
Corrected Total	23	1.3355			

R-Square	C.V.	Root MSE	RFW Mean
0.086893	90.20360	0.246932	0.27375000

Dependent Variable: RDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00069	0.00023	0.31	0.8144
Error	20	0.01478	0.00073		
Corrected Total	23	0.01548			

R-Square	C.V.	Root MSE	RDW Mean
0.045114	80.17574	0.027193	0.03391667

Dependent Variable: RDW/SDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.0625	0.02084	0.94	0.4403
Error	20	0.4439	0.02219		
Corrected Total	23	0.5065			

R-Square	C.V.	Root MSE	RDW/SDW Mean
0.123478	62.17285	0.148992	0.23964119

Dependent Variable: Change in Root Length (RL=RL2-RL1)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	9.3276	3.10922	0.30	0.8256
Error	20	207.8610	10.39305		
Corrected Total	23	217.1886			

R-Square	C.V.	Root MSE	RL Mean
0.042947	62.58336	3.223825	5.15125000

Dependent Variable: Change in Stem Length (SL=SL2-SL1)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	16.570	5.5233	0.75	0.5373
Error	20	148.069	7.4034		
Corrected Total	23	164.639			

R-Square	C.V.	Root MSE	SL Mean
0.100645	81.93523	2.720932	3.32083333

Dependent Variable: SSWC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1.2742	0.4247	1.16	0.3494
Error	20	7.3190	0.3659		
Corrected Total	23	8.5932			

R-Square	C.V.	Root MSE	SSWC Mean
0.148284	15.18724	0.604939	3.98320283

Dependent Variable: SRWC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	29.7625	9.9208	2.51	0.0881
Error	20	79.0810	3.9540		
Corrected Total	23	108.8435			

R-Square	C.V.	Root MSE	SRWC Mean
0.273443	32.57377	1.988480	6.10454292

Dependent Variable: PSDM

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	17.5118	5.8372	0.94	0.4407
Error	20	124.4408	6.2220		
Corrected Total	23	141.9527			

R-Square	C.V.	Root MSE	PSDM Mean
0.123364	12.25343	2.494403	20.3567682

Dependent Variable: PRDM

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	221.8249	73.941	1.67	0.2047
Error	20	883.7584	44.187		
Corrected Total	23	1105.5834			

R-Square	C.V.	Root MSE	PRDM Mean
0.200641	41.84414	6.647400	15.8860943

2. Experiment 2:

Dependent Variable: SFW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	2.7476	0.68691	3.51	0.0182
Error	30	5.86896	0.19563		
Corrected Total	34	8.61660			

R-Square	C.V.	Root MSE	SFW Mean
0.318878	41.72670	0.442303	1.06000000

Dependent Variable: SDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.1637	0.0409	2.76	0.0457
Error	30	0.4447	0.0148		
Corrected Total	34	0.6085			

R-Square	C.V.	Root MSE	SDW Mean
0.269152	53.36239	0.121758	0.22817143

Dependent Variable: RFW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0129	0.00322	0.07	0.9916
Error	30	1.4676	0.04892		
Corrected Total	34	1.4805			

R-Square	C.V.	Root MSE	RFW Mean
0.008722	61.24494	0.221182	0.36114286

Dependent Variable: RDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.0073	0.0018	2.06	0.1108
Error	30	0.0266	0.0008		
Corrected Total	34	0.0340			

R-Square	C.V.	Root MSE	RDW Mean
0.215621	69.31976	0.029827	0.04302857

Dependent Variable: RDW/SDW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.1432	0.0358	2.62	0.0549
Error	30	0.4108	0.0136		
Corrected Total	34	0.5540			

R-Square	C.V.	Root MSE	RDW/SDW Mean
0.258565	55.69743	0.117021	0.21010057

Dependent Variable: RL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	42.713	10.6784	1.50	0.2272
Error	30	213.582	7.1194		
Corrected Total	34	256.295			

R-Square	C.V.	Root MSE	RL Mean
0.166658	38.91155	2.668221	6.85714286

Dependent Variable: SL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	130.01588	32.5039	2.85	0.0411
Error	30	342.58011	11.4193		
Corrected Total	34	472.59600			

R-Square	C.V.	Root MSE	SL Mean
0.275110	75.42971	3.379251	4.48000000

Dependent Variable: SSWC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	3.2413	0.8103	0.21	0.9300
Error	30	114.9165	3.8305		
Corrected Total	34	118.1576			

R-Square	C.V.	Root MSE	SSWC Mean
0.027432	48.07452	1.957180	4.07113707

Dependent Variable: SRWC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	16.7536	4.1884	0.25	0.9065
Error	30	499.6778	16.6559		
Corrected Total	34	516.4314			

R-Square	C.V.	Root MSE	SRWC Mean
0.032441	48.88659	4.081167	8.34823518

Dependent Variable: PSDM

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	248.3347	62.0836	1.39	0.2625
Error	30	1344.0394	44.8013		
Corrected Total	34	1592.3742			

R-Square	C.V.	Root MSE	PSDM Mean
0.155953	31.01154	6.693378	21.5835112

Dependent Variable: PRDM

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1862.4644	465.616	3.14	0.0284
Error	30	4442.2198	148.073		
Corrected Total	34	6304.6843			

R-Square	C.V.	Root MSE	PRDM Mean
0.295410	86.87248	12.16857	14.0073893