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In vitro studies on germination of immature ovules and plant regeneration from cotyledons of *Impatiens platypetala* Lindl.

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In vitro studies on germination of immature ovules and plant regeneration from cotyledons of *Impatiens platypetala* Lindl.

by

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GENERAL INTRODUCTION

The technology of plant tissue culture has been developed to become a powerful tool for both basic and applied plant science (Williams et al. 1987). Among many other uses, the application of this technology to plant breeding has come to include the rescue of interspecific hybrids using embryo and ovule cultures (Hu and Wang 1986, Williams et al. 1987). The advances in tissue culture techniques should generate a number of new and potential applications for embryo and ovule cultures which have not been possible, as well as improve the efficiency of interspecific hybrid rescue (Williams et al. 1987, Bhojwani and Razdan 1983).

The most important aspect in improving interspecific hybrid rescue is to define a culture medium formulation which is able to support growth and development of immature embryos and ovules (Bhojwani and Razdan 1983). One reason for culturing immature embryos of interspecific hybrids is endosperm failure in developing ovules after fertilization (Bhojwani and Razdan 1983, Hu and Wang 1986). Arisumi (1980a) reported the presence of aborted embryos from several incompatible crosses among Impatiens species in the early developmental stages. Arisumi (1980b, 1985) tried to rescue the aborted interspecific hybrid embryos, but he obtained very low germination percentages of embryos and ovules. According to other reports (Williams et al. 1987, Hu and Wang 1986,

Bhojwani and Razdan 1983), medium requirements for embryos and ovules depended upon their maturity at excision, and younger embryos and ovules required more complex medium than did mature ones. The results from previous studies (Arisumi 1980b, 1985) suggested that the composition of the culture medium was not optimum for germination of embryos and ovules of Impatiens.

Plant regeneration from mature or immature cotyledonary explants has been successful during the last decade in many horticultural crop species such as peanut (McKently et al. 1990, Atreya et al. 1984), apple (Korban and Skirvin 1985), Japanese pear (Hiratsuka and Katagiri 1988), Citrus (Gmitter and Moore 1986), pecan (Yates and Wood 1989), and Cucumis melo (Dirks and van Buggenum 1989), and in some other agriculturally important crops such as Glycine max (Lippmann and Lippmann 1984, Mante et al. 1989), Helianthus (Bohorova et al. 1985), Brassica juncea (Fazekas et al. 1986), etc. However, plant regeneration via organogenesis, embryogenesis, or any other regeneration method has not been developed yet for any Impatiens species. Thus, cotyledons were studied as an explant to establish an efficient plant regeneration system for Impatiens species. An efficient system of plant regeneration is necessary for genetic transformation using Agrobacterium, the system of choice for dicot species (Horsch et al. 1985, An et al. 1986)). Thus, successful Impatiens plant regeneration should make it possible to transform

Impatiens via Agrobacterium-mediated transformation, thereby adding another promising tool to Impatiens breeding programs.

Explanation of Dissertation Format

This dissertation is arranged in the alternate format consisting of two papers that will be submitted to scientific journals. Kyungchul Han was the principal investigator on all research reported herein, and he is the first author on both papers. Dr. Loren C. Stephens served as the major Professor for Kyungchul in his research and is listed as an author on both papers.

LITERATURE REVIEW

In vitro Germination of Embryo and Ovule Cultures

Carbohydrates as a Carbon Energy Source and as an Osmoticum

A carbohydrate such as sucrose, glucose, fructose, etc. is usually added to the culture medium as an energy source for immature embryo and ovule cultures. Among the carbohydrates, sucrose is most commonly used as a carbon energy source (Williams et al. 1987, Hu and Wang 1986).

Wang and Janick (1986) reported that zygotic embryo growth of cacao was optimal in the medium supplemented with 9% sucrose and was poor in 3% sucrose. Mannitol reduced embryo growth in semisolid medium which was the osmotic equivalent of 21% sucrose. Sucrose, glucose or fructose all supported the growth of asexual embryos, but glucose was clearly superior to sucrose in the growth and development of asexual embryos of cacao (Kononowicz and Janick 1984). It was also observed that dry and fresh weights of asexual embryo of cacao were greater in 13.5% than in 27% glucose and sucrose. Growth inhibition brought about by increased sugar concentration (27%) might be due to the increased osmotic potential of the medium or by oxygen limitation (Kononowicz and Janick 1984).

Chung et al. (1988) used sucrose at concentrations ranging from 4 to 10% in rescuing tobacco interspecific hybrids through ovule culture. In subsequent experiments, a part of sucrose was replaced with mannitol to adjust the osmotic

potential of the medium containing 2, 3, or 4% sucrose to that equivalent to 6 to 10% sucrose. They obtained higher germination rates of 5-day-old hybrid ovules cultured at the carbohydrate concentrations equivalent to 7 to 9% sucrose than other concentrations tested. They theorized that sucrose in this range fulfilled both nutritional and osmoregulative functions and a concentration of at least 3 to 4% sucrose was necessary for the growth of the embryo as a nutrient source and another 4 to 5% carbohydrate as an osmoticum. However, Tukey (1934) reported in deciduous fruit species that 2% glucose was beneficial for the culture of young zygotic embryos, but was inhibitory for more mature embryos. The requirement of more than 2% sucrose by the immature embryos was osmotically related rather than being a metabolic need (Hu and Wang 1986).

In addition to the nutritional requirement, carbohydrate might be required to maintain suitable osmotic potential of the culture medium. The osmotic potential of the embryo sac environment varies from high values (e.g., about 10% sucrose equivalent) to lower values (2% sucrose) as the embryo enlarges. Thus, an increasing osmotic potential may be required to induce physiological maturity of an immature embryo (Wang and Janick 1986). The concentration required for embryos and ovules depends upon the ages of ovules and embryos at excision (Williams et al. 1987, Hu and Wang 1986). Mannitol is often used to maintain high osmotic potential of

the culture medium. Although mannitol has been frequently used as a metabolically inert osmoticum in plant tissue culture, its toxicity has been reported in embryo culture (Pretova 1974).

Ryczkowski (1962a,b) observed that during the early stages of embryo development the central vacuolar sap of the ovule showed greater osmotic value than during later stages of development, but with further growth of the embryo, the value decreased considerably. Ryczkowski (1969) later found that the increase of osmotic value of the central vacuolar sap to a determined maximum and its subsequent drop takes place during the inhibition phase of the growth of the embryo and during the first part of the exponential phase of the growth of ovules in all species tested. The changes in osmotic value of the central vacuolar sap were observed in the period of intensive growth and development of the endosperm tissue up to a complete disappearance of the central vacuole. Embryos in the exponential phase of growth have a higher osmotic value, compared with the surrounding endosperm tissue, or the central vacuolar sap. The liquid endosperm in which young embryos are constantly bathed generally had more negative osmotic potential values and culture media with relatively more negative osmotic potential were beneficial to the growth of young embryos in culture (Raghavan 1976). Young developing seeds constantly maintained more negative osmotic potentials, which indicated that the developing embryos of Phaseolus

vulgaris were part of an osmotic gradient and favored water movement into the seed (Yeung and Brown 1982). Thus, an environment with a more negative osmotic potential value may, at least in part, play a regulatory role in the development of the embryo in vivo and in vitro. Thus, osmotic conditions of young immature ovules and embryos should be optimized by modifying the composition of the culture medium when culturing them for germination.

Inorganic Nitrogen Source

The utilization of inorganic nitrogen in embryo and ovule cultures differs by plant species as well as by ages of embryos and ovules (Hu and Wang 1986). Ammonium (NH_4^+) nitrogen in the culture medium was either essential or preferred for proper growth and differentiation of immature barley embryos (Umbeck and Norstog 1979) and immature embryos of Datura tutula (Matsubara 1964), whereas nitrate (NO_3^-) nitrogen was preferred by Cruciferae (Rijven 1958). However, two forms of nitrogen, ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3), have been commonly used in combination as sole sources of inorganic nitrogen in embryo and ovule cultures (Hu and Wang 1986). Bhojwani and Razdan (1983) also suggested using ammonium and nitrate nitrogen in combination because of dramatic pH changes of the medium containing NH_4Cl alone even though ammonium form (NH_4Cl) alone as a sole source of nitrogen was needed for embryogenesis.

Shoot Organogenesis from Cotyledons

Organogenesis in tissue cultures refers to the direct formation of plant organs such as shoots, roots, leaves, etc. from parental explants during culture. Organogenesis is different from embryogenesis in that organogenic tissue is monopolar and connected with the vascular system of the explant, while embryogenic tissue is bipolar and is not connected with the vascular system (Haccius 1978).

Plant regeneration from cotyledonary explants has been successfully achieved in plant species such as Arachis hypogaea (McKently et al. 1990, Atreya et al. 1984), Cucumis melo (Dirks and Buggenum 1989), Pinus strobus (Kaul 1987), pear (Browning et al. 1987), Brassica juncea (Fazekas et al. 1986), Japanese pear (Hiratsuka and Katagiri 1988), interspecific hybrids between Camellia vietnamensis and C. chrysantha (Nadamitsu et al. 1986) and Glycine max (Lippmann and Lippmann 1984). An efficient plant regeneration system can be important for clonal propagation of favorable lines through in vitro culture of explants and recently has become important for genetic transformation studies using Agrobacterium (Horsch et al. 1985) as tools for plant breeding programs.

McKently et al. (1990) established a plant regeneration system by culturing several types of cotyledon explants obtained from peanut seeds on MS medium supplemented with BA.

Maximal production of multiple shoots was obtained on a medium supplemented with 25 mg/l BA. Atreya et al. (1984) also reported a successful regeneration system from both embryo axes and cotyledonary segments of peanut cultured on MS medium supplemented with 2 mg/l BA. Shoot induction occurred predominantly from the cotyledonary segments proximal to the embryo axes. They hypothesized that plant regeneration potential decreases from the proximal to the distal region of a cotyledon, and a gradient of regeneration potential exists within a cotyledon.

In plant regeneration in vitro, polarity or orientation of cotyledon explants affects the regeneration potential during culture. In their studies on morphogenesis in embryonic tissue cultures of apple, Rubos and Pryke (1984) found that the petiole of the cotyledon was most responsive in morphogenesis among the different parts of the embryo tissue and the initiation of shoots and roots was localized only at the petiole of the cotyledon. Adventitious organs formed on the petioles of apple cotyledons originated from epidermal cells. Kouider et al. (1984) observed a number of significant differences among the various cotyledon excision treatments for multiple shoot formation from apple cotyledons. They reported that adventitious multiple shoot formation occurred only at the proximal end of an excised cotyledon. In other words, shoot organogenesis occurred only at the proximal cut surfaces of both proximal and distal explants rather than at

the distal cut surfaces of proximal explants. These results indicate the presence of an unidirectional polarity in plant regeneration of cotyledon explants. However, shoot organogenesis from the proximal cut surface of distal cotyledon explants is in contrast with the results reported by Rubos and Pryke (1984) that shoot regeneration occurred only in tissues close to or derived from the proximal petiole of the cotyledon.

Browning et al. (1987) reported that a higher frequency of shoot organogenesis was obtained from the distal cut surfaces of proximal cotyledon explants than from the proximal cut surfaces of distal explants. They hypothesized that multiple shoot regeneration was independent of the basipetal orientation and an unidirectional polarity of the explant in shoot organogenesis. Their hypothesis is in direct contrast with that reported by Kouider et al. (1984). Browning et al. (1987) also reported that multiple shoot regeneration was enhanced when the cut surfaces of the vertically oriented proximal or distal explants were maintained above, rather than immersed into the medium. A similar result was reported by Zimmerman and Fordham (1989) that more axillary shoots developed from subcultured shoots of apple that were placed inverted in the medium. By contrast, Gertsson (1986) reported that shoot formation was most prolific on the petiole explants of Senecio x hybridus grown base downwards, whereas the formation of roots and callus was more prolific on the

explants grown upwards. Similarly, others have reported that shoot formation occurred mainly at the morphological bases of the explants, regardless of explant orientation (Harney and Knap 1979, Welander 1981).

These conflicting studies make it necessary to examine conditions for *in vitro* germination of immature ovules and for plant regeneration via shoot organogenesis from cotyledonary explants, but there are common factors that seem to affect *in vitro* germination such as carbohydrate type, carbohydrate concentration, inorganic nitrogen, and explant age. Common factors that frequently affect shoot organogenesis are auxin, cytokinin, explant type, and explant age. The factors in each of these two systems, *in vitro* germination and shoot organogenesis, are what I intend to optimize in my studies with *Impatiens*.

SECTION I. FACTORS AFFECTING *IN VITRO* GERMINATION OF IMMATURE OVULES OF *IMPATIENS PLATYPETALA* LINDL.

FACTORS AFFECTING *IN VITRO* GERMINATION OF IMMATURE OVULES OF *IMPATIENS PLATYPETALA* LINDL.

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ABSTRACT

The effects of carbohydrates, inorganic nitrogen, and explant age on *in vitro* germination of immature ovules of *Impatiens platypetala* Lindl. 'TR6-27-4' and 'TR6-27-2' were investigated. Carbohydrate type and concentration affected *in vitro* germination of immature ovules. In its overall effects on germination, glucose was less inhibitory than was sucrose or mannitol, and a carbohydrate concentration at 0.025 M was also less inhibitory than higher concentrations. There were no differences in germination among the 3 carbohydrates at 0.025 M. The age of immature ovules affected germination and subsequent seedling growth differently at different sucrose concentrations. Inorganic nitrogen concentration did not affect germination regardless of ovule age, but seedling fresh weight was significantly less and abnormal development of seedlings was significantly increased by total inorganic nitrogen concentrations higher or lower than 30 mM (at a ratio of 20:10 mM NO_3^-:NH_4^+) in the culture medium.

INTRODUCTION

After Java and New Guinea Impatiens, I. platypetala Lindl. and I. hawkeri Bull. respectively, were introduced into the United States in 1970 by USDA plant explorers (Winters 1973), Java and New Guinea Impatiens and other species have been crossed to obtain interspecific hybrids (Arisumi 1974, 1975, 1978, 1980a,b, 1985, 1987, Beck et al. 1974, Pasutti et al. 1977, Pasutti and Weigle 1980, Weigle and Pasutti 1979a,b, and Weigle et al. 1979).

Most embryos formed by these intercrosses aborted in the early developmental stages within 2 weeks after pollination, about 2-3 weeks before the normal maturation period (Arisumi 1980a). Arisumi (1980b) attempted to apply embryo and ovule culture methods to overcome the barriers of interspecific sterility and to rescue the aborted embryos of Impatiens hybrids. He obtained low germination of embryos and ovules from self- and cross-pollinated Impatiens, and few germinated embryos and ovules were recovered as seedlings (Arisumi 1985). Arisumi (1985) also attempted similarly to rescue abortive Impatiens hybrids through aseptic culture of immature ovules. He reported that none of the ovules germinated in 28 crosses and only 0.3 to 40% of the ovules successfully germinated in another 38 crosses (Arisumi 1985). These results led us to suspect the culture medium as a possible cause of the relatively poor germination.

Several preliminary experiments indicated that sucrose and inorganic nitrogen in the culture medium were inhibiting *in vitro* germination and causing abnormal seedling growth respectively, after germination of immature ovules. Thus, the objectives of this research were to determine the effects of carbohydrates and inorganic nitrogen in MS medium on *Impatiens* ovule germination *in vitro*. An inbred line of Java *Impatiens* was used to limit genotypic variation, so that a medium could be developed that could be tested on other species and interspecific hybrids in the future.

MATERIALS AND METHODS

Preparation of Explant

Two Impatiens platypetala genotypes, 'TR6-27-2' and 'TR6-27-4', were chosen as plant materials because they are self-compatible and have better seed set than New Guinea Impatiens (I. hawkeri). Both genotypes are full siblings that are inbreds of P.I. 349629, an I. platypetala accession from the island of Java, Indonesia, that was brought back to the USA by USDA plant explorers in 1970 (USDA 1972, Winters 1973). All plants were grown in the Iowa State University (ISU) Department of Horticulture research glasshouse and used as parental plants to obtain immature ovules after self-pollination. All plants of each genotype were grown in a mixture of 2/3 sphagnum peat moss: 1/3 horticultural grade vermiculite, with the addition of ground limestone to raise the pH to 6.0. All plants of each genotype were fertilized once a week with 100 ppm N of a 20-16.6-8.8 (% N-P-K) analysis water-soluble fertilizer.

Flowers were emasculated by removing the anther hood before the stigma was receptive. Self-pollinations were made by applying pollen from a freshly opened flower to receptive stigmas and the date of pollination was then recorded. Fruits were harvested within 12 to 20 days after pollination, or about 10-20 days before the normal maturation period, to obtain different ages of immature ovules. The fruits were

surface-disinfested by dipping in 70% ethanol for approximately 30-60 seconds followed by dipping in 0.5% NaOCl (10% v/v commercial laundry bleach) for 15 minutes and rinsed 4 times with sterile deionized water. Aseptically, 2 to 3 mm from both ends of the fruits were cut off, then they were cut open longitudinally to extract the ovules inside the fruits. The ovules were detached with forceps and 5 ovules were plated onto culture medium in sterile petri dishes. The petri dishes were sealed with parafilm and incubated in growth chambers.

Preparation of Medium

A basal medium was composed of inorganic MS salts (Murashige and Skoog, 1962), with the addition of 100 mg/liter myo-inositol, 0.5 mg/liter nicotinic acid, 0.5 mg/liter pyridoxine·HCl, 0.1 mg/liter thiamine·HCl, 2.0 mg/liter glycine and 30 g/liter sucrose. According to the objectives of each experiment, several components in the carbohydrate and nitrogen portion of the basal medium were modified. Several concentrations of sucrose, glucose, and mannitol were used in place of 30 g/liter sucrose as modifications to the carbohydrate portion of the basal medium. Nitrogen in the basal medium was modified by varying the concentrations of total inorganic nitrogen from that of the basal medium. In a separate experiment, factorial nitrogen treatments consisting of 3 levels (0, 20, and 40 mM) of NO_3^- and 3 levels (0, 10, and 20 mM) of NH_4^+ were made by replacing NH_4NO_3 with $NaNO_3$ and

by using $(NH_4)_2SO_4$ and KCl to find the effects of NH_4^+ and NO_3^-. The pH of all prepared media was adjusted to 5.78-5.82 before adding 5 g/liter Difco Bitek-agar. The media were autoclaved 15 minutes at 1.1 Kg cm^{-2} (124 kPa) and 121° C and then approximately 25 ml of each medium was dispensed into sterile petri dishes.

Culture Conditions and Statistical Analyses

The petri dishes with plated ovules were incubated in a growth chamber set at 26-28° C under 16 hours of illumination of approximately 135 to 190 μmols $m^{-2}sec^{-1}$ with Cool White fluorescent lamps (General Electric, Inc.).

The experiments were designed with factorial treatments in a completely random design (CRD). Depending upon the experimental situations and explant availability, each experiment was composed of 5 to 24 treatments, and each treatment was composed of 7 to 10 replications (petri dishes). Each petri dish contained 5 ovules. Data for percent germination, percent abnormality, and fresh weight per seedling were collected after 5 to 6 weeks incubation. Abnormality was defined as abnormal seedling morphology after germination, including albino, yellowish green, or yellowish brown cotyledons or seedlings. Data were statistically analyzed by using the General Linear Models Procedure of the Statistical Analysis System (SAS Institute, Inc., 1987) at the end of each experiment. F-tests, regression analyses,

multiple range tests (LSD) and/or other statistical analyses were applied to interpret the data from each experiment.

RESULTS

In vitro germination of 20-day-old immature ovules of genotype 'TR6-27-4' was significantly inhibited, depending on carbohydrate type and concentration (Table I-1). The linear regression effect of carbohydrate concentrations was significant at the 1% level (Table I-1, Fig. I-1). Thus, as carbohydrate concentration increased, the germination percentage decreased proportionately. The 3 carbohydrates showed no differences for percent germination at 0.025 M, but germination was inhibited by sucrose or mannitol at 0.05 M, whereas inhibition did not occur until the concentration of glucose was raised to 0.1 M. (Fig. I-1). Sucrose and mannitol affected *in vitro* germination of immature ovules similarly at each concentration tested (Fig. I-1).

For genotype 'TR6-27-2', the germination percentage on 0.025 M sucrose was greater than that on 0.1 M sucrose, regardless of the ages of immature ovules tested (Table I-2, Fig. I-2). The germination percentage on 0.025 M sucrose increased with increasing age of immature ovule, but on 0.1 M sucrose 12-day-old ovules germinated significantly better than 14- and 16-day-old ovules (Table I-2, Fig. I-2).

Among the 3 ages of immature ovules tested, 16-day-old ovules showed more vigorous seedling growth than 14- and 12-day-old immature ovules after germination on 0.025 M sucrose, whereas there was no significant difference in seedling growth

on 0.1 M sucrose, regardless of ovule ages tested (Table I-2, Fig. I-3).

Nitrogen concentration was not significantly different for in vitro germination of immature ovules (Table I-2), and the mean percentages of germination were 39, 46, and 46 for 0, 30, and 60 mM nitrogen, respectively, for ovules 12 to 16 days old. The percent of abnormal seedlings was significantly greater after germination on 60 mM nitrogen than on 0 and 30 mM nitrogen, regardless of ovule age (Table I-2, Fig. I-4). Seedlings from 14- and 16-day-old ovules had significantly more abnormalities than those from 12-day-old ovules after germination on 60 mM nitrogen. There were no significant differences in the percent of abnormal seedlings on 0 or 30 mM nitrogen, for seedlings from ovules of all 3 ages (Fig. I-4). Sucrose at 0.025 M combined with the highest inorganic nitrogen concentration (60 mM) produced the most abnormal seedlings, whereas 0.1 M sucrose combined with 60 mM inorganic nitrogen had significantly fewer abnormal seedlings (Table I-2, Fig. I-5). The percent of abnormal seedlings was not significantly different for 0 or 30 mM nitrogen, regardless of whether the sucrose concentration was 0.025 or 0.1 M (Table I-2, Fig. I-5). In a separate experiment, the effects of various ratios of NO_3^-/NH_4^+ on subsequent seedling growth after germination of 17 day-old immature ovules were significant at the 5% level, according to an F-test (not shown). A single degree-of-freedom F-test indicated that the seedling growth

was greatest when nitrogen in the basal MS medium was replaced with 20 mM NO_3^- and 10 mM NH_4^+, compared with all other treatments consisting of various NO_3^-/NH_4^+ ratios tested (Fig. I-6).

Table I-1. Analysis of variance for the effects of carbohydrate types and their concentrations on the *in vitro* germination of 20 day-old immature ovules of *Impatiens platypetala* 'TR6-27-4' on agar-solidified MS medium after 5 weeks in culture.

Source	DF	SS	F	PR>F
Replication	4	8349.70	3.73	0.0103*
Carbohydrate	3	19064.09	11.37	0.0001**
Concentration	4	46125.70	23.62	0.0001**
Effects due to regression				
Linear[z]	1	45717.54	46.83	0.0001**
Quadratic	1	408.16	0.42	0.5204
Carb*concentration	6	8263.91	2.46	0.0378*
Error	46	25719.13		

*P<0.05.

**P<0.01.

[z]Linear equation, Y=98-840X.

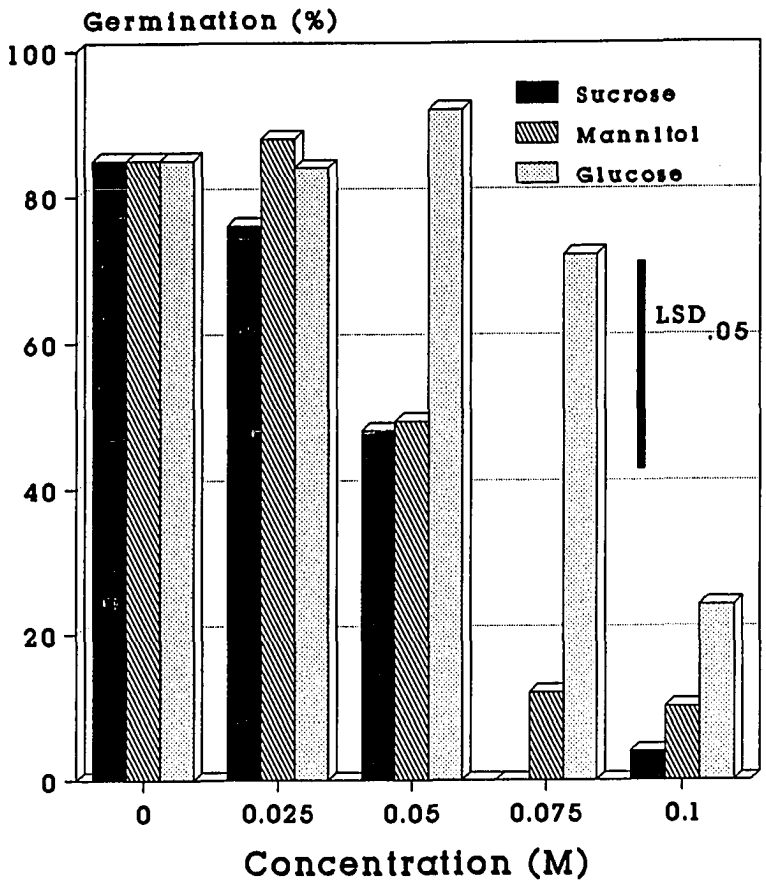

Fig. I-1. Interaction of carbohydrate type with carbohydrate concentration on the means of percent germination of 20 day-old immature ovules of Impatiens platypetala 'TR6-27-4' after 5 weeks in culture.

Table I-2. Analysis of variance for the effects of sucrose and inorganic nitrogen concentration on the means of percent germination, seedling growth (fresh weight), and percent abnormality[z] for 3 ages (12, 14, and 16 day-old) of immature ovules of Impatiens platypetala 'TR6-27-2' on agar-solidified MS medium after 6 weeks in culture.

Source	DF	SS			PR>F		
		Germ	FW	ABN	Germ	FW	ABN
Replication	9	5154.70	13624.50	4765.30	0.346	0.289	0.405
Age	2	4085.69	29029.35	2109.67	0.020*	0.001**	0.128
Sucrose	1	82217.31	21830.70	3169.77	0.001**	0.001**	0.013*
Nitrogen	2	2044.44	1333.11	40798.93	0.137	0.586	0.001**
Age*sucrose	2	10156.52	31853.41	1421.02	0.001**	0.001**	0.248
Age*nitrogen	4	1646.63	2622.76	8705.57	0.520	0.716	0.003**
Suc*nitrogen	2	1675.47	6306.24	6916.33	0.195	0.083	0.001**
Age*suc*nitro	4	3675.15	13599.83	2200.33	0.130	0.031*	0.365
Error	152	77160.85	189207.34	76844.85			

[z]Abnormality was defined as abnormal seedling morphology after germination, including albino, yellowish green, or yellowish brown cotyledons or seedlings.

*$P<0.05$.

**$P<0.01$.

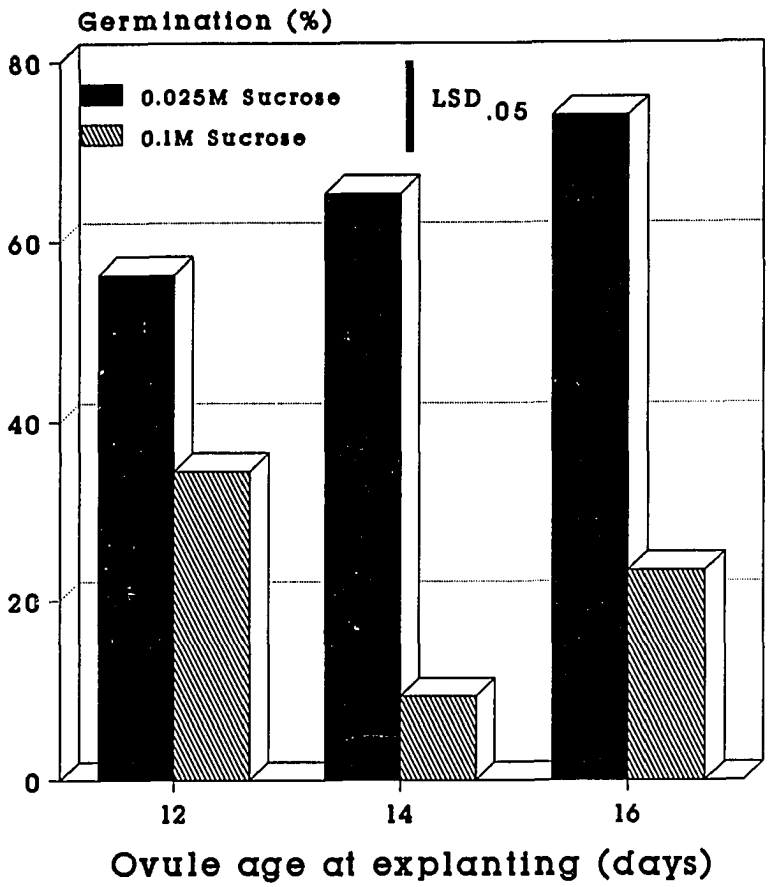

Fig. I-2. Interaction between explant ages and sucrose concentrations on the means of percent germination of immature ovules of Impatiens platypetala 'TR6-27-2' on agar-solidified MS medium after 6 weeks in culture.

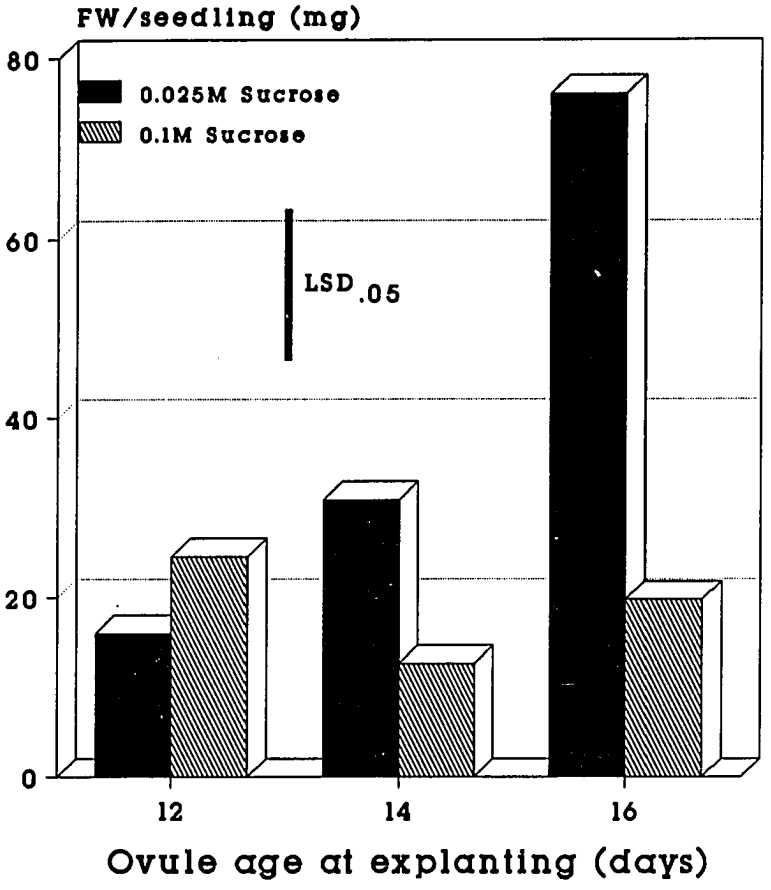

Fig. I-3. Interaction between explant ages and sucrose concentrations on the means of fresh weight of germinated seedlings from immature ovules of Impatiens platypetala 'TR6-27-2' on agar-solidified MS medium after 6 weeks in culture.

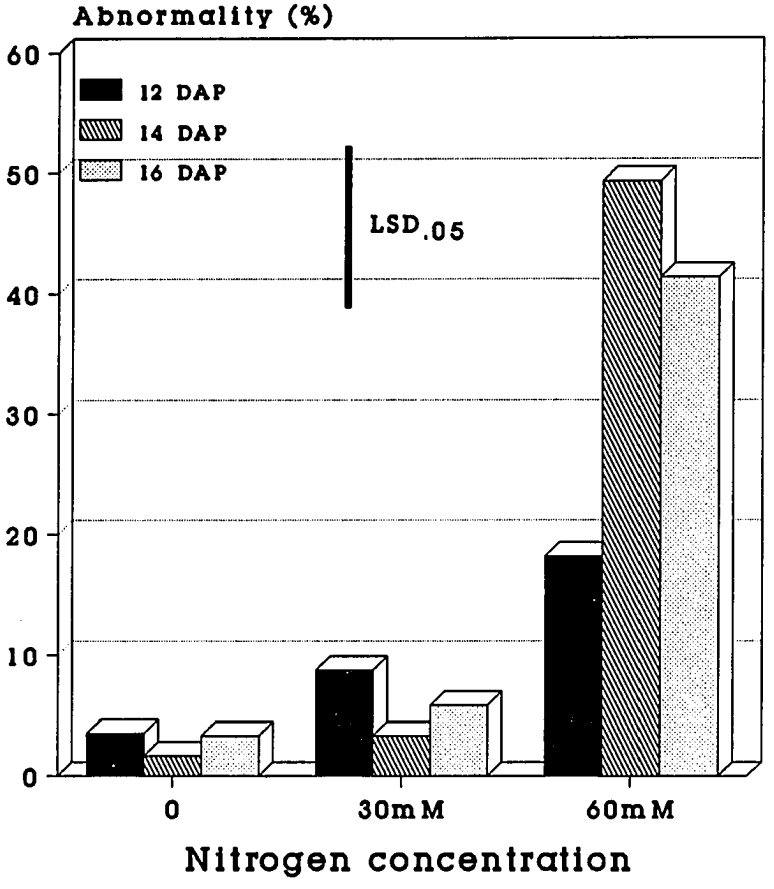

Fig. I-4. Interaction between inorganic nitrogen concentration and ovule age on the mean percent production of abnormal seedlings of Impatiens platypetala 'TR6-27-2' in agar-solidified MS medium after 6 weeks in culture.

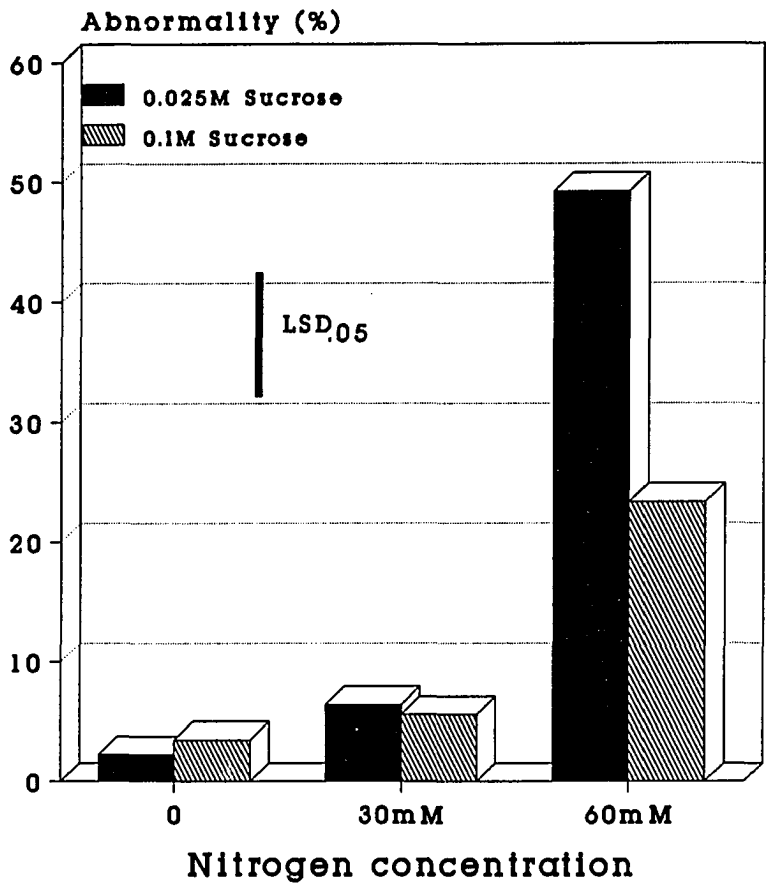

Fig. I-5. Interaction between sucrose and nitrogen concentrations on the mean percent production of abnormal seedlings of Impatiens platypetala 'TR6-27-2' in agar-solidified MS medium after 6 weeks in culture.

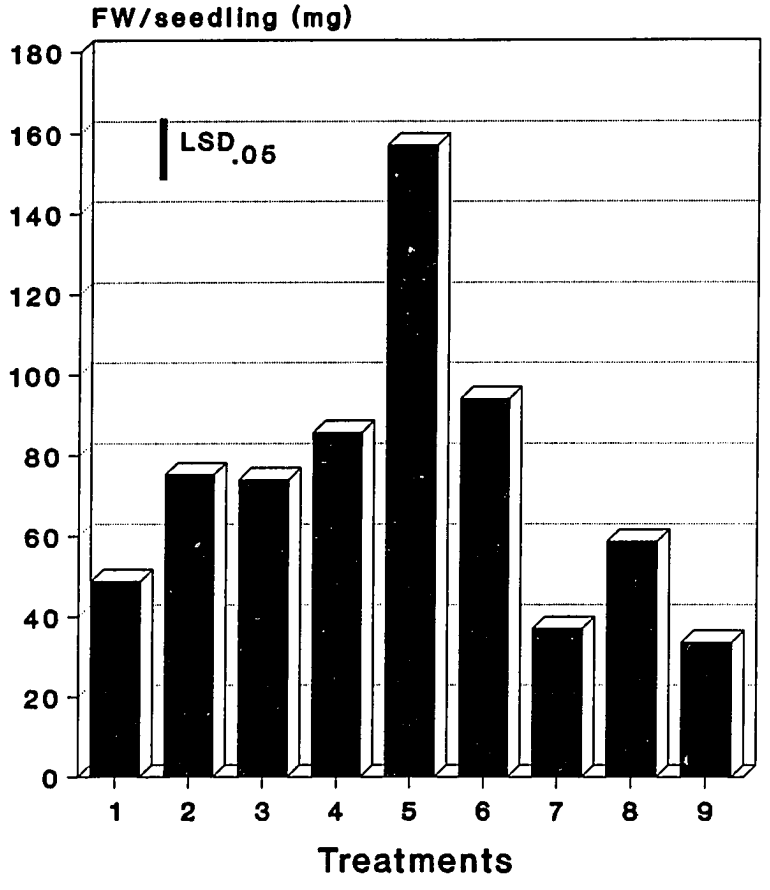

Fig. I-6. Effects of inorganic nitrogen forms on the means of fresh weight of germinated seedlings from 17 day-old immature ovules of Impatiens platypetala 'TR6-27-2' after 7 weeks in culture. Treatments, 1 through 9, were composed of various NO_3^-/NH_4^+ ratios, 0/0, 0/10, 0/20, 20/0, 20/10, 20/20, 40/0, 40/10, and 40/20 mM, respectively. Single degree-of-freedom F-test showed that "Treatment 5 vs all other treatments" was significant at the 1% level.

DISCUSSION

The results presented here, that *in vitro* germination of immature ovules of *Impatiens* was significantly inhibited by increasing carbohydrate concentration greater than 0.05 M sucrose or mannitol, were similar to other reports for orchid germination (Pierik et al. 1988). In contrast, several other researchers reported that higher carbohydrate concentrations were required for younger embryos and ovules (Chung et al. 1988, Antonelli et al. 1988, Niederwieser et al. 1990). The concentration required for embryos and ovules depended largely upon the ages of embryos and ovules at excision (Williams et al. 1987, Hu and Wang 1986). Pro-embryos of *Ornithogalum* required a relatively high sucrose concentration (70 g/liter or approx. 0.2 M) to survive and this requirement decreased to 10 g/liter as embryos developed (Niederwieser et al. 1990). Chung et al. (1988) also reported that sucrose concentrations ranging from 6 to 8% (or approx. 0.18-0.24 M) in the culture medium were optimal for the culture of the pro-embryo stage of immature hybrid ovules of *Nicotiana*. They found that a concentration of at least 3-4% (approx. 0.09-0.12 M) sucrose was necessary for the growth of the embryos as a nutrient source and the remainder was needed as an osmoticum. However, this study showed that the percent germination of *Impatiens* ovules of all 3 ages examined was significantly lower on 0.1 M than on 0.025 M sucrose even though younger ovules (12-day-

old) germinated better than 14- and 16-day-old ovules on 0.1 M sucrose (Fig. I-2). Unlike the reports that an increasing osmotic potential may be required to induce physiological maturity and better germination of immature ovules and embryos (Wakizuka and Nakajima 1974, Wang and Janick, 1986, Chung et al. 1988), this study suggests that an increasing osmotic potential of the culture medium is inhibitory for in vitro germination of 12 to 16 day-old ovules of Impatiens and a carbohydrate concentration lower than 0.05 M is needed. This suggestion can be supported by noting that both mannitol, a metabolically inert carbohydrate that acts as an osmoticum and not as an energy source in the culture medium, and sucrose, inhibited germination at a concentration higher than 0.05 M (Fig. I-1). Arisumi (1980b, 1985) obtained poor germination of immature Impatiens ovules and embryos ranging from 5 to 27 days after pollination by an addition of 20 to 40 g/liter sucrose (or approx. 0.06 to 0.12 M) to the culture medium. The poor germination may be partially the result of the sucrose concentrations used in his studies.

In this study, glucose was less inhibitory than sucrose and mannitol for germination of 20-day-old immature ovules of genotype 'TR6-27-4'. A similar result was reported for asexual embryos of Theobroma cacao (Kononowicz and Janick 1984). Welander et al. (1989) reported that glucose was most effective among 5 carbon sources, sucrose, glucose, fructose, mannitol and sorbitol, for in vitro shoot multiplication from

bud explants of Alnus glutinosa. Tremblay and Lalonde (1984) also found similar effects with glucose for in vitro propagation of 6 Alnus species tested whereas sucrose was superior to glucose for A. glutinosa. The requirement of a certain type of carbohydrate might be explained by differences in plant species and the ability of a given species to metabolize a carbohydrate. This explanation was advanced by Zimmermann and Ziegler's findings (1975), in which carbohydrate analyses of sieve-tube exudates showed significant differences in the carbohydrates that could be metabolized among plant families.

The age of immature Impatiens ovules at excision was also an important factor affecting germination. Percent germination increased as the age of ovules of genotype 'TR6-27-2' increased from 12 to 16 days after pollination. Similar results have been reported in several other plants (Antonelli et al. 1988, Chung et al. 1988, Niederwieser et al. 1990). Niederwieser et al. (1990) used embryos as explants ranging from 4 to 24 days (mature cylindrical embryos) after pollination and found that an ability of Ornithogalum embryos to grow in vitro increased with embryo age. In ovule culture of interspecific hybrids of Nicotiana, 5-day-old hybrid ovules germinated better than 3- and 4-day-old ovules on medium with sucrose ranging from 4 to 10% (0.12 to 0.3 M) (Chung et al. 1988).

In this study, inorganic nitrogen affected subsequent

seedling growth after germination rather than affecting germination itself. Abnormal development of seedlings germinated *in vitro* from immature ovules has also been reported in *Impatiens* (Arisumi 1985). In his study (Arisumi 1985), he found that approximately 75% of germinated seedlings developed abnormally and the abnormal development was caused by cultural and genetic deficiencies. However, abnormal seedlings may have been caused by high ammonium nitrogen because it is known that seedlings utilize NH_4^+ faster than NO_3^- (Rahman 1988). Plantlets transferred from *in vitro* to *ex vitro* conditions were affected by nitrogen, particularly in the form of NH_4^+, during the early stages of establishment of *Artocarpus* (Rahman 1988). Weigle et al. (1982) reported ammonium toxicity on rooted cuttings of *Impatiens platypetala* with varying NH_4^+/NO_3^- ratios. According to their results, plants receiving excess NH_4^+ had more mishappen leaves than those in other treatments. The results presented here suggest that both forms of inorganic nitrogen are necessary for optimal seedling growth. It seems likely that total nitrogen concentrations lower than 30 mM caused nitrogen deficiency in seedling growth, whereas nitrogen concentrations higher than 30 mM may have resulted in nitrogen toxicity.

Immature ovules of *Impatiens platypetala* genotypes, 'TR6-27-4' and 'TR6-27-2', can be successfully germinated *in vitro* by modifying carbohydrate concentration and type, and inorganic nitrogen strength in a MS-based medium.

Specifically, MS medium should be modified by decreasing sucrose concentration to 0.025 M (approx. 8 g/liter) and by decreasing the nitrogen concentration to 30 mM, with 10 mM being ammonium nitrogen and 20 mM being nitrate nitrogen.

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SECTION II. *IN VITRO* PLANT REGENERATION FROM COTYLEDONS OF IMMATURE OVULES OF *IMPATIENS PLATYPETALA* LINDL. 'TR6-17-2'

IN VITRO PLANT REGENERATION FROM COTYLEDONS OF IMMATURE OVULES OF *IMPATIENS PLATYPETALA* LINDL. 'TR6-27-2'

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ABSTRACT

An efficient and reproducible protocol has been developed for plant regeneration from cotyledonary explants derived by *in vitro* germination of immature *Impatiens* ovules (*I. platypetala* Lindl. 'TR6-27-2'). Cotyledonary explants were cultured *in vitro* for 6-8 weeks on a modified Murashige and Skoog (MS) agar medium containing 7.5 g/liter sucrose, 5 mg/liter N^6-benzyladenine (BA), and 0.1 mg/liter α-naphthalene acetic acid (NAA). This medium is designated as organogenic tissue induction (OTI) medium to induce organogenic tissues from explants. Exogenous auxin (NAA) and cytokinin (BA) were essential for OTI and survival of explants, and BA was most effective for OTI, compared with other cytokinins tested. Glutamine (500 mg/liter) supplemented to OTI medium was also important for the growth of organogenic tissues after OTI and for reducing explant death during culture. OTI was also influenced by explant type and age of donor seedlings germinated *in vitro*. An average of 7.6 shoots per explant was induced from organogenic tissues larger than 0.5 cm in diameter 6-8 weeks after transfer to a modified MS agar medium containing 7.5 g/liter sucrose and 1 mg/l BA (SI medium). Shoots longer than 0.5 cm in length were successfully rooted 2-4 weeks after transfer to a basal MS medium containing 30 g/liter sucrose.

INTRODUCTION

An introduction of new traits into plants by means of genetic transformation has recently opened up a new era for plant breeding and crop improvement programs (Old and Primrose 1989, Puonti-Kaerlas et al. 1990). To accomplish this, an efficient plant regeneration system via shoot organogenesis or embryogenesis is highly desirable, particularly for genetic manipulation using the Agrobacterium-mediated transformation system (Horsch et al. 1985). This study was undertaken because no regeneration system currently exists for Impatiens.

Impatiens has become one of the most popular ornamental bedding plants in the United States, according to industry sources such as Bedding Plants, Inc. (Dr. David Koranski, personal communication). After the introduction of Indonesian and New Guinea Impatiens into the United States in 1970 (USDA 1972, Winters 1973), research has been undertaken to improve New Guinea Impatiens by conventional plant breeding methods (Pasutti and Weigle 1980, Arisumi 1974, 1978, 1980, 1987), but conventional breeding programs have limitations that make it difficult to combine all desirable traits into one cultivar. Often, an otherwise popular cultivar has one or two undesirable traits. Such a problem may be possible to overcome by Agrobacterium-mediated transformation, which is able to transfer a specific foreign gene into a plant (Wullems et al. 1986, An et al. 1986, Chee et al. 1989, Puonti-Kaerlas

et al. 1990, Rhodes et al. 1988). This system may be applied to Impatiens after a plant regeneration system is established because Impatiens is a dicotyledonous plant species and thus should be susceptible to Agrobacterium infection.

Mature or immature cotyledons have been successfully used as an explant to obtain plant regeneration in many horticultural crop species such as peanut (McKently et al. 1990, Atreya et al. 1984), apple (Korban and Skirvin 1985), Japanese pear (Hiratsuka and Katagiri 1988), Citrus (Gmitter and Moore 1986), pecan (Yates and Wood 1989), and Cucumis melo (Dirks and van Buggenum 1989), etc. Thus, because all these plants, like Impatiens, are dicots, cotyledons would seem a good candidate to use as an explant to establish a plant regeneration system for Impatiens.

The objective of this research was to find and establish an efficient plant regeneration system for Impatiens that maximizes both consistency and efficiency of plant regeneration as well as the number of plants attainable from cotyledonary explants.

MATERIALS AND METHODS

Preparation of Plant Materials

Impatiens platypetala Lindl. 'TR6-27-2' was chosen as plant material because it is self-compatible and has better seed set than its cultivated relative, New Guinea Impatiens (I. hawkeri Bull.). 'TR6-27-2' is an inbred selection derived from P.I. 349629, an I. platypetala accession from the island of Java, Indonesia, that was brought back to the USA by USDA plant explorers in 1970 (USDA, 1972, Winters 1973). All cloned plants were grown in the Iowa State University (ISU) Department of Horticulture research glasshouse and used as stock plants to obtain immature ovules after hand pollination. All plants of this genotype were grown in a mixture of 2/3 sphagnum peat moss: 1/3 horticultural grade vermiculite, with the addition of ground limestone to raise the pH to 6.0. All plants were fertilized once a week with 100 ppm N of a 20-16.6-8.8 (% N-P-K) analysis water-soluble fertilizer.

Flowers were emasculated by removing the anther hood before the stigma was receptive. Self-pollinations were made by applying pollen from a freshly opened flowers to receptive stigmas and the date of pollination was then recorded. Fruits were harvested within 18-20 days after pollination, or about 1-2 weeks before the normal maturation period. The fruits were surface-disinfested by dipping in 70% ethanol for approximately 30 seconds followed by dipping in 0.5% NaOCl

(10% v/v commercial laundry bleach) for 15 minutes and rinsed 4 times with sterile deionized water. Aseptically, 2 to 3 mm from both ends of the fruits were cut off, then they were cut open longitudinally to extract the ovules inside the fruits with forceps.

Preparation of Medium

A basal medium was composed of inorganic MS salts (Murashige and Skoog, 1962), with the addition of 100 mg/liter myo-inositol, 0.5 mg/liter nicotinic acid, 0.5 mg/liter pyridoxine·HCl, 0.1 mg/liter thiamine·HCl, 2.0 mg/liter glycine and 30 g/liter sucrose. According to the objectives of each experiment, several components in the basal medium were modified or added. The pH of the medium was adjusted to 5.78-5.82 before adding 5 g/liter Difco-BiTek agar. The media were autoclaved 15 minutes at 1.1 Kg cm^{-2} (124 kPa) and 121° C and then approximately 25 ml of each medium was dispensed aseptically into sterile 100 mm petri dishes.

In Vitro Germination

The basal medium was modified by changing to 7.5 g/liter sucrose and by adding 1 mg/liter BA, and 500 mg/liter glutamine to obtain in vitro germination of immature ovules. Aseptically, 31 detached ovules were plated onto the culture medium. The petri dishes with plated ovules were sealed with parafilm and incubated in growth chambers for 2-3 weeks.

Cotyledons from germinated seedlings *in vitro* were selected and used as the explants in this study.

Induction of Organogenic Tissue

Healthy green and cup-shaped cotyledons with thickened petioles from *in vitro* germination were selected and used as explants and the cotyledon base attached to the petiole was cut off (Fig. II-2a). It was not necessary to sterilize the plant materials before plating the explant onto the culture medium because the selected cotyledons were apparently pathogen-free.

In all experiments for the induction of organogenic tissues, 7.5 g/liter sucrose was added to the basal culture medium. Depending upon the objectives of each experiment, the basal medium was modified differently. NAA combined with several concentrations of cytokinins (BA, Kinetin, and 2iP) were added into the basal medium to determine the best combination for organogenic tissue induction (OTI). Glutamine (500 mg/liter) and/or casein hydrolysate (500 mg/liter) were added into the basal medium containing 0.1 mg/liter NAA and 5 mg/liter BA, hereafter designated as organogenic tissue induction medium (OTIM). In separate experiments, 3 different cotyledon explants (whole cotyledon, distal half, and proximal half of a cotyledon) and 3 different ages of cotyledon explants (13, 17, and 21 days after plating immature ovules onto *in vitro* germination medium) were used as treatments and

were plated onto OTIM containing 500 mg/liter glutamine. Cultures were incubated in growth chambers for 6-8 weeks until data were collected.

Induction of Shoots and Roots

To induce shoot development, 10-12 explants producing organogenic tissues greater than 0.5 cm in diameter were sampled from several experiments using OTIM with or without 500 mg/liter glutamine. These explants were transferred to the basal medium containing 7.5 g/liter sucrose, 1 mg/liter BA, and 500 mg/liter glutamine, hereafter designated shoot induction (SI) medium. Data for shoot number per explant were taken after 6-8 weeks in culture and plant conversion rate (Y) was modified from the criterion for somatic embryogenesis (Eltjo et al. 1987) and calculated by the equation, Y = total number of explants producing shoots divided by total number of explants sampled and transferred to SI medium, and this result was then multiplied by 100 to obtain a percent conversion.

For root development, agar-solidified basal medium (rooting medium) was prepared to induce roots from shoots. Shoots longer than 0.5 cm and with more than 4 leaves were cut off from organogenic tissues producing shoots in SI medium and transferred onto the rooting medium.

Culture Conditions and Statistical Analyses

The petri dishes with plated explants were incubated in the growth chambers set at 26-28° C under 16 hours of illumination of approximately 135-190 μmols $m^{-2}sec^{-1}$ with Cool White fluorescent lamps (General Electric, Inc.). The cultures were incubated for 2-8 weeks, depending upon objectives of each experiment.

All experiments were designed as a completely random design (CRD) with factorial treatments. Depending upon the experimental situations and explant availability, each experiment was composed of 3 to 15 treatments, and each treatment was composed of 7 to 10 replications (petri dishes). Each petri dish contained 5 cotyledon explants. Data for the number of explants producing organogenic tissue, diameter of organogenic tissue, the number of explants producing callus, and the number of dead explants were taken 6 to 8 weeks after incubation. Organogenic tissue was defined as a dark green, well-organized tissue that was morphologically distinct from non-organogenic tissue, which was a light green to yellowish white, friable callus. Both types of tissue formed mainly around the cut base of explants. Data were statistically analyzed by using the General Linear Models Procedure of the Statistical Analysis System (SAS Institute, Inc. 1987) at the end of each experiment. F-tests, regression analyses, multiple range tests (LSD) and/or other statistical analyses were applied to interpret the data from each experiment.

RESULTS

Cotyledon explants formed organogenic tissue after 6-8 weeks of culture on OTI medium (Fig. II-2b). The effects of cytokinin type on percent organogenic tissue induction (OTI), percent callus formation, percent explant death, and diameter of organogenic tissue were significant at the 1% level, according to an F-test (not shown). Single degree-of-freedom F-tests indicated that BA induced greater percent OTI than either kinetin or 2iP (Table II-1). Compared with other cytokinins tested, presence of BA also resulted in the least explant death and the largest diameter of organogenic tissue (Table II-1). Kinetin resulted in the poorest OTI and the greatest explant death, whereas 2iP induced more callus than did the other two cytokinins tested (Table II-1).

Regression analysis showed that the percent of OTI was greatest at 5 mg/liter BA and there was a lesser percent of OTI at BA concentrations higher and lower than 5 mg/liter (Table II-2). The percent of callus formation increased linearly as BA concentrations were increased in the medium, whereas the percent of explant death was significantly less for 5 mg/liter BA, compared with 1 mg/liter BA (Table II-2). OTI was not affected by NAA concentrations tested, but 0.5 mg/liter NAA resulted in more callus formation and less explant death than did 0.1 mg/liter NAA (Table II-2).

The effects of glutamine and casein hydrolysate (C.H.) on

percent OTI, percent callus formation, and percent explant death were significant at the 1% level, according to an F-test (not shown). Single degree-of-freedom F-tests indicated that the percent of OTI was significantly greater when 500 mg/liter glutamine was added to OTI medium, compared with OTI medium itself (Table II-3). However, the percent of OTI was significantly less when either 500 mg/liter C.H. or 500 mg/liter glutamine and 500 mg/liter C.H. were added to OTI medium, compared with OTI medium with or without glutamine (Table II-3). Percent callus formation was significantly greater when either C.H. or glutamine and C.H. were added to OTI medium, compared with OTI medium with glutamine (Table II-3). Percent explant death was significantly lower when 500 mg/liter glutamine was added into OTI medium, compared with OTI medium itself (Table II-3).

The effects of explant type on percent OTI, percent callus formation, percent explant death, and diameter of organogenic tissue were significant at the 1 or 5% level, according to an F-test (not shown). Single degree-of-freedom F-tests indicated that greater percent OTI was obtained from whole and proximal half of cotyledons than from distal half, and whole cotyledon explants were not significantly different from proximal-half explants for percent OTI (Table II-4). Compared with distal-half explants, whole and proximal-half explants also resulted in fewer explant deaths and a larger diameter of organogenic tissue (Table II-4). The percent of OTI from

whole and proximal-half explants was greater at 5 mg/l BA than at 1 mg/l BA, whereas organogenic tissues were not induced from distal-half cotyledon explants at any BA concentration tested (Fig. II-1).

The effects of explant age of cotyledons on percent OTI, diameter of organogenic tissue, and percent callus formation were significant at the 1% level, according to an F-test (not shown). Single degree-of-freedom F-tests indicated that a greater percent OTI and a lesser percent callus formation were obtained from 13 and 17 day-old cotyledons than from 21 day-old cotyledons (Table II-5). Compared with other explant ages tested, 13 day-old cotyledons resulted in the largest diameter of organogenic tissues, whereas there was no significant difference in percent explant death from all explant ages tested (Table II-5).

Among 42 explants of organogenic tissues transferred to SI medium from OTIM with glutamine, 29 explants developed multiple shoots (Fig. II-2c, Table II-6), and the plant conversion rate was 69% (Table II-6). The mean shoot number per explant of organogenic tissue producing multiple shoots was 7.6 (Table II-6). Shoots longer than 0.5 cm in length with more than 4 leaves were transferred to the basal medium containing 30 g/liter sucrose, where they rooted successfully during 2-4 weeks of culture after transfer (Fig. II-2d).

Table II-1. Effects of 5 mg/liter of 3 cytokinins combined with 0.1 mg/liter NAA on the mean percentages of organogenic tissue[x] induction (OTI), callus formation (CF), explant death (ED), and the mean diameter of organogenic tissue (DOT) of cotyledon explants of Java Impatiens platypetala 'TR6-27-2'.

Cytokinins	Variables			
	OTI[y](%)	CF(%)	ED(%)	DOT(mm)
BA	67.27	12.73	20.00	4.72
Kinetin	7.27	3.64	89.09	1.27
2iP	17.87	37.78	44.44	2.09
[z]$LSD_{.05}$	14.64	12.23	17.94	1.68

[x]Organogenic tissue was defined as a dark green, well-organized tissue that was morphologically distinct from non-organogenic tissue, which was a light green to yellowish white, friable callus; both types of tissue formed mainly around the cut base of explants.

[y]Single degree-of-freedom F-tests: BA vs Kinetin+2iP**, Kinetin vs 2iP^{ns} (^{**,ns}significant at the 1% level and not significant, respectively).

[z]Mean comparison in column by LSD test at the 5% level.

Table II-2. Effects of BA combined with NAA in MS medium containing 7.5 g/liter sucrose on the mean percentages of organogenic tissue induction (OTI), callus formation (CF), and explant death (ED) of cotyledon explants of Java Impatiens platypetala 'TR6-27-2'.

Hormone	Level (mg/l)	Variables		
		OTI(%)	CF(%)	ED(%)
NAA	0.1	22.75	2.50	73.75
	0.5	25.00	25.00	50.00
	$LSD_{.05}$	10.60	11.52	15.44
	1	20.00	2.50	77.50
BA	5	40.00	15.00	45.00
	10	25.00	12.50	62.50
	15	12.50	25.00	62.50
	$^{W}LSD_{.05}$	14.99	16.29	21.84
Effect due to regression		Q^{X}	L^{Y}	NS^{Z}

[W]Mean comparison in column by LSD test at the 5% level.

[X]Quadratic effect due to regression at the 1% level. Regression equation, $Y=-0.4X^2+5X+18$.

[Y]Linear effect due to regression at the 5% level. Regression equation, $Y=1.4X+3$.

[Z]Not significant.

Table II-3. Effects of 500 mg/liter glutamine (Gln) and 500 mg/liter casein hydrolysate (C.H.), in OTI medium (OTIM) on the mean percentages of organogenic tissue induction (OTI), callus formation (CF), and explant death (ED) of cotyledon explants of Java Impatiens platypetala 'TR6-27-2'.

Medium	Variables[y]		
	OTI(%)	CF(%)	ED(%)
Control (OTIM)	50.00	0.00	50.00
OTIM+Gln	86.67	6.67	6.67
OTIM+C.H.	16.67	63.33	16.67
OTIM+Gln+C.H.	33.33	56.67	10.00
[z]$LSD_{.05}$	25.15	23.05	27.30

[y]Single degree-of-freedom F-tests: Control vs Gln for OTI**, Gln vs C.H. and Gln+C.H. for CF**, Control vs Gln for ED** (**significant at the 1% level).

[z]Mean comparison in column by LSD test at the 5% level.

Table II-4. Effects of cotyledon explant types plated onto OTI medium containing 500 mg/liter glutamine on the mean percentages of organogenic tissue induction (OTI), callus formation (CF), explant death (ED), and mean diameter (mm) of organogenic tissue (DOT) of cotyledon explants of Impatiens platypetala 'TR6-27-2'.

Explant type	Variable[y]			
	OTI(%)	CF(%)	ED(%)	DOT(mm)
Whole cotyledon	41.25	20.00	38.75	4.06
Proximal half	42.22	28.89	26.67	4.13
Distal half	0.00	17.78	82.22	0.00
[z]$LSD_{.05}$	10.37	10.14	11.66	0.13

[y]Single degree-of-freedom F-tests: Whole and proximal half vs distal half for OTI**, ED**, and DOT** (**significant at the 1% level).

[z]Mean comparison in column by LSD test at the 5% level.

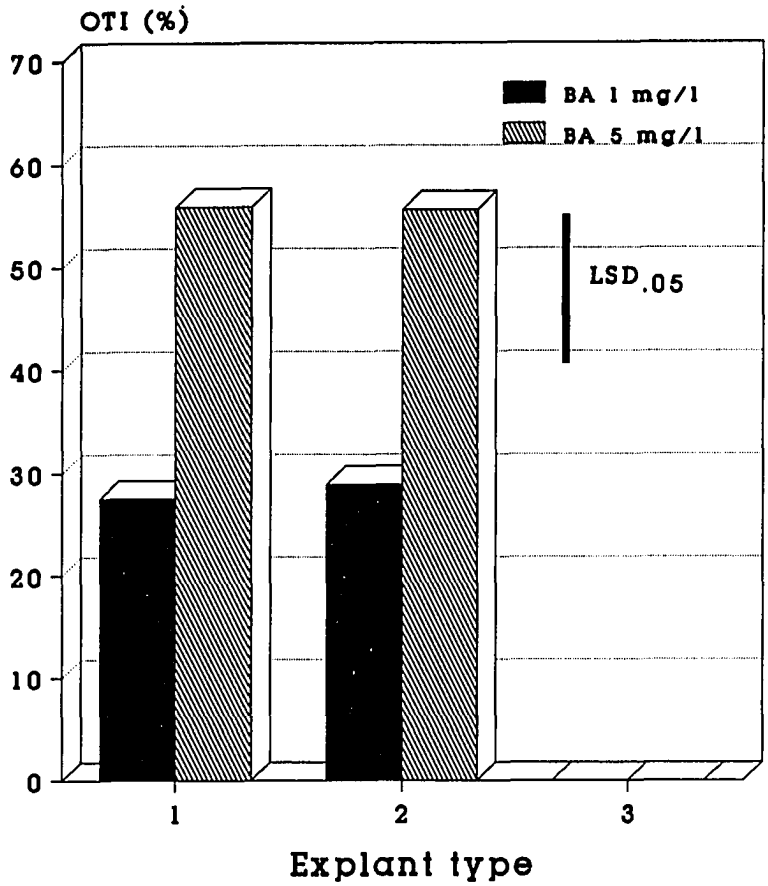


Fig. II-2. Interaction of BA and explant type in OTI medium containing 500 mg/liter glutamine on organogenic tissue induction of 3 cotyledon explants (1: whole, 2: proximal half, 3: distal half) of Impatiens platypetala 'TR6-27-2'.

Table II-5. Effects of explant ages of cotyledons after plating 19 day-old immature ovules for germination on the mean percentages of organogenic tissue induction (OTI), callus formation (CF), explant death (ED), and the mean diameter (cm) of organogenic tissue (DOT) from cotyledon explants of Impatiens platypetala 'TR6-27-2' in OTI medium containing 500 mg/liter glutamine.

Cotyledon age	Variable[y]			
	OTI(%)	DOT(cm)	CF(%)	ED(%)
13 day-old	70.77	0.42	0.00	29.23
17 day-old	56.92	0.33	9.23	33.85
21 day-old	20.00	0.27	53.33	26.67
[z]$LSD_{.05}$	18.29	0.07	17.45	23.71

[y]Single degree-of-freedom F-tests: 13 and 17 vs 21 day-old for OTI** and CF** (**significant at the 1% level).

[z]Mean comparison in column by LSD test at the 5% level.

Table II-6. Multiple shoot induction after 6-8 weeks in culture from organogenic tissues produced by several experiments in agar-solidified MS medium containing 7.5 g/liter sucrose, 1 mg/liter BA, and 500 mg/liter glutamine (SI medium).

Expt. No.	No. of explants sampled[w]	No. of EPS[x]	Total shoot no.	Mean SNPE[y]
SU-90-4	10	6	29	4.8
SU-90-7	10	8	73	9.1
SU-90-12	10	7	69	9.9
F-90-2	12	8	49	6.1
Total	42	29	220	7.6
Plant conversion rate[z] = 100 x 29/42 = 69 (%)				

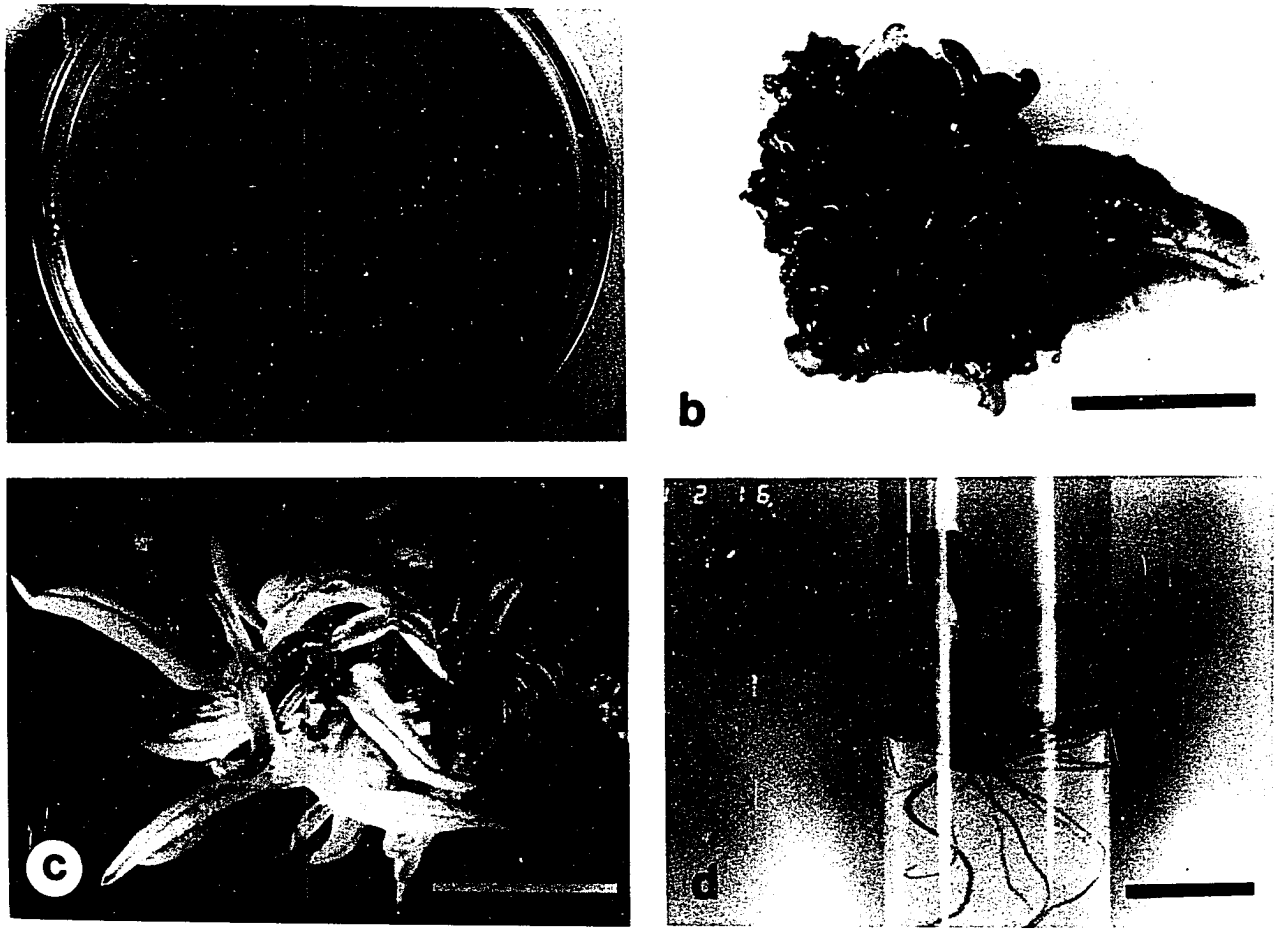
[w]Number of explants producing organogenic tissues greater than 0.5 cm in diameter sampled to transfer to multiple shoot induction (SI) medium.

[x]Explants producing shoot.

[y]Shoot numbers per explant.

[z]Conversion rate (%) was defined as total no. of EPS divided by total no. of explants transferred x 100.

Fig. II-2. (a) Explant one week after plating, (b) explant forming organogenic tissue, (c) multiple shoots from organogenic tissue, (d) shoots with roots. The length of each bar is 1 cm.


b
c
d

DISCUSSION

BA at 5 mg/liter was most effective for OTI of Impatiens 'TR6-27-2' cotyledon explants. Gertsson (1986) reported that BA was a more effective cytokinin than zeatin, kinetin, or 2iP for stimulating shoot formation from petiole explants of Senecio x hybridus. Other studies showed that the optimal BA concentration varied from 1 to 25 mg/liter, depending upon the plant species (McKently et al. 1990, Atreya et al. 1984, Hiratsuka and Katagiri 1988, Dirks and van Buggenum 1989, Fazekas et al. 1986, Nadamitsu et al. 1986, Kouider et al. 1984a,b, Browning et al. 1987). For Impatiens 'TR6-27-2', BA concentrations higher and lower than 5 mg/liter were not preferable for plant regeneration of Impatiens because BA concentrations higher than 5 mg/liter stimulated callus formation and rather inhibited OTI, whereas BA concentrations lower than 5 mg/liter significantly delayed OTI and the overall processes of plant regeneration.

Both NAA and BA were needed for OTI of Impatiens 'TR6-27-2' because all cotyledon explants turned light to dark brown on the culture medium not supplemented with either NAA or BA, and finally died after 3-4 weeks of culture. Kim et al. (1990) reported that 0.02 mg/liter NAA was supplemented to the culture medium not because it stimulated shoot formation from explants of primary leaf nodes of soybean seedlings, but because shoots formed on the medium with NAA became stronger

than those on the medium without it. In contrast, Kaul (1987) reported that the supplement of 0.02 mg/liter NAA into the culture medium was necessary for better shoot induction from cotyledon-hypocotyl explants of Pinus strobus. The findings of Browning et al. (1987) were similar to those of this study, in that the culture medium supplemented with both 0.1 mg/liter NAA and 5 mg/liter BA was most effective for shoot organogenesis from cotyledon explants of pear embryos.

The addition of glutamine into OTI medium stimulated greater OTI of Impatiens 'TR6-27-2' cotyledon explants as well as decreased percent explant death during culture. Bohorova et al. (1985) reported that more shoots were produced from the shoot-tip explants of Helianthus species when 800 mg/liter glutamine and 800 mg/liter asparagine were added into shoot-inducing medium. Similar results were also obtained by the addition of 2 mg/l proline for plant regeneration from primary leaf nodes of soybean seedlings (Kim et al. 1990). For Impatiens 'TR6-27-2', the addition of 500 mg/liter glutamine into the germination medium also increased the number of available cotyledons for explants from the germinated seedlings (unpublished data).

Only whole and proximal-half cotyledon explants of Impatiens 'TR6-27-2' formed organogenic tissues and calli from the cut bases, whereas distal-half explants either died or did not respond during the culture incubation period. Similar results were reported for peanut cotyledon explants (Atreya et

al. 1984). Such differences in response among explant types suggest that a unidirectional polarity exists in the cotyledon explants, in which a certain gradient of plant regeneration potential exists from the proximal to the distal region within a cotyledon. This polarity was also observed in apple cotyledons (Kouider et al. 1984b, Rubos and Pryke 1984). Kouider et al. (1984b) hypothesized that the polarity might be the result of a topological, developmental, morphological, chemical, or a combination of some or all of these factors, whereas Gertsson (1986) theorized that the polarity might be related to the concentrations of plant growth regulators, mainly cytokinins and auxins, supplemented to the culture medium. In contrast, Browning et al. (1987) reported that any strict unidirectional polarity was not observed from pear embryo cotyledon explants, and plant regeneration was independent of the basipetal orientation of the explants.

For Impatiens 'TR6-27-2', age of cotyledon explants was also an important factor affecting OTI and plant regeneration. Hiratsuka and Katagiri (1988) reported similarly, that the age of cotyledon explants significantly affected callus and shoot formation in Japanese pear. In their study (Hiratsuka and Katagiri 1988), callus and shoot formation was obtained only from the explants between 11 and 12 weeks after pollination, and no shoot formation was observed from explants younger than 11 weeks and older than 12 weeks after pollination. Explant age was also found to affect regeneration for Glycine max (Kim

et al. 1990) and for strawberry (Nehra et al. 1989).

Although a mean of 7.6 shoots per explant were produced from all organogenic tissue explants, it should be possible to obtain more shoots per explant by subculturing organogenic tissues more than 2 times, because organogenic tissues continued growing in size as well as developing multiple shoots in the SI medium.

In vitro plant regeneration of Impatiens platypetala 'TR6-27-2' was successfully accomplished, using cotyledons as explants from in vitro germination of immature ovules. The plant regeneration system established for Impatiens in this study is summarized in Fig. II-2. Cotyledon explants from in vitro germination of immature ovules were plated onto agar-solidified OTI medium supplemented with 500 mg/liter glutamine in sterile 100 mm petri dishes and cultured for 6-8 weeks. Organogenic tissues larger than 0.5 cm were transferred to SI medium and cultured for another 6-8 weeks to develop shoots. Multiple shoots longer than 0.5 cm were transferred to agar-solidified basal medium to induce roots. Shoots successfully rooted within 2-4 weeks after transfer.

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SUMMARY AND DISCUSSION

Several factors affecting *in vitro* germination of immature ovules and plant regeneration from cotyledons of *Impatiens* were investigated. Carbohydrate type, carbohydrate concentration, inorganic nitrogen, and explant age were studied as important factors affecting *in vitro* germination. Concentrations of NAA and BA, additions of glutamine and/or casein hydrolysate, explant type, and explant age from germinated seedlings were studied as important factors involved in shoot organogenesis.

There were no differences in percent germination among the 3 carbohydrates at 0.025 M. However, germination was inhibited by sucrose or mannitol at 0.05 M, whereas inhibition did not occur until the concentration of glucose was raised to 0.1 M. *In vitro* germination of all 3 ages of ovules showed greater inhibition on 0.1 M sucrose than on 0.025 M sucrose. The age of immature ovules affected germination and subsequent seedling growth differently at different sucrose concentrations. Inorganic nitrogen did not affect *in vitro* germination itself but abnormal development of seedlings was significantly increased by total inorganic nitrogen concentrations higher or lower than 30 mM (at ratios other than 20:10 mM NO_3^-:NH_4^+) inorganic nitrogen forms in the culture medium.

The greatest percent of organogenic tissue from

cotyledonary explants of Impatiens was induced on a modified MS medium containing 7.5 g/liter sucrose, 0.1 mg/liter NAA, and 5 mg/liter BA designated as organogenic tissue induction (OTI) medium after 6-8 weeks in culture. NAA at 0.1 mg/liter was more effective for OTI than at 0.5 mg/liter, and among the 3 cytokinins tested, BA was most effective for OTI. Both NAA and BA were essential for OTI and for the survival of cotyledonary explants. Glutamine (500 mg/liter) supplemented to OTI medium was also necessary for the growth of organogenic tissues and for increasing the survival of cotyledonary explants.

Based upon these findings for OTI, it seemed desirable to determine the effects of explant type and age of cotyledons derived from in vitro germination on OTI. Whole and proximal-half cotyledons were more effective for OTI than was distal-half cotyledons and a certain gradient of regeneration potential similar to an unidirectional polarity was observed among 3 explant types. Explant age of cotyledons was also an important factor affecting OTI and plant regeneration. A greater OTI was obtained from 13 and 17 day-old cotyledons than from 21 day-old cotyledons.

Explants of organogenic tissue induced on OTI medium with glutamine developed multiple shoots 6-8 weeks after transfer to SI medium. Eight shoots per explant were formed. Shoots were successfully rooted 2-4 weeks after transfer to a basal MS medium containing 30 g/liter sucrose.

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