INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2005/07–4–621–626
http://www.ijab.org

Effect of Different Plant Growth Regulators for the Economical Production of *in vitro* Root Cultures of *Cicer arietinum* L.

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ABSTRACT

Root organ cultures of chickpea (*Cicer arietinum* L.) were developed under *in vitro* conditions to study the effect of various growth regulators on rapid propagation and multiplication of roots. Root explants of *Cicer arietinum* L. were cultured on half and full strength Murashige and Skoog (MS) medium with different concentrations (0.25-1.00 mg L^{-1}) of indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP). IBA in a concentration of 0.5 and 0.75 mg/L and BAP in the concentrations of 0.25, 0.75 and 1.00 mg/L resulted in moderate rooting response (++) after five weeks of culturing while very little rooting (+) was achieved with the addition of IAA after five weeks. Different concentrations of NAA and 2,4-D resulted in callogenesis rather than the induction of roots. Between NAA and 2,4-D, 2,4-D was found better for the induction of callus. Different combinations of IAA and IBA, and IBA and NAA resulted in adequate root culture response. 1.00 mg/L of both IBA+NAA and 1.00 mg/L of both IAA+IBA resulted in adequate rooting (+++) after six week of culturing. Among the media, half strength MS solid medium was found to be better for root production as compared to full strength MS solid medium. Although, addition of different concentrations and combinations of growth hormones in the medium affect the growth of roots but their lack from the MS medium resulted in excellent root formation. Best root culture was achieved in half strength liquid MS medium lacking hormones.

Key Words: *Cicer arietinum* L.; Growth regulators; Half and full strength MS medium; *In vitro* root cultures
Abbreviations: 2,4-D = 2,4-Dichlorophenoxyacetic acid; BAP = 6-Benzylaminopurine; IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; KN = Kinetin; MS = Murashige and Skoog; NAA = 1-Naphthaleneacetic acid

INTRODUCTION

Tissue culture involves the production of plants from very small plant parts, tissues, or cells, grown aseptically in a test tube or other container where the environment and nutrition can be rigidly controlled (Hartmann & Dale, 1983). The different types of culture can be classified into organ cultures, callus culture, meristem and tissue culture, somatic embryogenesis, cell culture and protoplast culture etc. Organ cultures are the aseptic cultures of embryos, anthers, ovaries, buds, roots, flower and other plant organs to form plantlets in media, which usually contain plant hormones such as auxins and cytokinins or some times free of hormones (Bajaj, 1990; Anju & Chawla, 2005). The tissue culture technique used to get virus free plants has been extended to some grain legumes (Bajaj, 1990). The successful regeneration of entire plantlets was observed in *Cicer arietinum* L. (Chaturvedi & Chand, 2001).

in vitro root cultures of chickpea are obtained by germinating explants in MS medium. Shoot tips (Polisetty *et al*., 1996), roots, cotyledonary nodes and apexes are taken from seedlings obtained from culturing of embryos from mature seeds, and used as explant. These explants are transferred on MS medium containing IBA (Jia & Eaton, 1984), BAP (Fernandez *et al*., 1998), IAA and KN for root production (Fratini & Ruiz, 2003) and on MS medium devoid of cytokinins but containing 2,4-D or NAA for callus production (Morjane & Harrabi, 1993). Nodal segments of chickpea in inverted position resulted in good root cultures on MS medium containing growth regulators (Fratini & Ruiz, 2003). Growth regulators have major effect on callus and root cultures in chickpea. Regeneration and hairy root of different types of chickpea have been developed using callus-derived plantlets and seedlings (Altinkut *et al*., 1997).

Different strengths of MS medium are used for obtaining root cultures. Half and quarter strength MS media are better for *in vitro* rooting than full strength MS medium (Polisetty *et al*., 1996). Both solid and liquid MS media are used. Solid and liquid media have significant difference with respect to rooting. For chickpea, liquid media has efficient growth of roots with respect to solid media, which has lesser growth rate (Fernandez *et al*., 1998).

Root cultures can be used in many ways including studies of carbohydrate metabolism, mineral nutrient requirements, essentially of vitamins and other growth regulators, differentiation of the root apex and gravitropism. The advantage of using root cultures is that they grow rapidly, are relatively easy to prepare and maintain, show a slow level of variability and can be easily cloned to produce a large supply of experimental tissues (Stribling, 1983).

Root culture is used to meet the continuous supply of secondary metabolites of nutritional and medicinal importance. Recently, many pharmaceutical companies are producing root cultures for making the metabolites, which are used in manufacturing of drugs (Hamil *et al.*, 1987).

The present study was undertaken to develop root culture by growing seedlings of chickpea under *in vitro* conditions, taking roots as explants and growing them on solid and liquid MS media. The effect of various growth regulators was investigated on rapid propagation and multiplication of roots. Large numbers of diseased free roots were produced by organ culture, which can be used for the production of important metabolites.

MATERIALS AND METHODS

Plant material. The plant material used to develop root culture were seeds of chickpea (*Cicer arietinum* L.) procured from NARC, Islamabad.

Medium composition and preparation. Basal medium used in the present study was Murashige and Skoog (MS) (1962) medium. Stock solutions of macronutrients, micronutrients, iron source and organic supplements were prepared separately for the preparation of MS medium. Sucrose (2%) was added to the medium as the basic energy source and pH of medium was adjusted to 5.7-5.8 with the addition of 1*N* HCl / NaOH. Agar (0.8%) was added to the medium, as solidifying agent and medium was autoclaved at 121ºC for 20 min at 15 lb psi pressure.

Seed sterilization and germination. The chickpea (*Cicer arietinum* L.) seeds were soaked in sterilized distilled water for 6-8 h and then surface sterilized with 0.1% mercuric chloride solution for 2-3 min. The seeds were thoroughly washed 4-5 times with autoclaved distilled water and testa was removed in laminar airflow hood (Dalton Corporation, Japan). The seeds were immediately transferred into a test tube containing 0.8% plan agar and kept in growth room at 28ºC in darkness for germination. After 24 h, the germinating seedlings were exposed 18/6 light/dark cycle (Fratini & Ruiz, 2003).

Explant inoculation and culture conditions. Lateral roots of 0.2-0.3 cm pieces of one-week old seedling cultured aseptically were served as explants for the root culture. Explants were transferred to flasks each containing MS medium along growth hormones. These flasks were placed in a temperature-controlled room at 25 ± 2ºC with 18/6 h light/dark cycle. Relative humidity in the culture room was 75 ± 5 % and light intensity was maintained at 2000 lux throughout the experiment (Puangpaka *et al*., 2001).

Growth regulators. Effects of various growth regulators were determined on rapid propagation and multiplication of roots in MS basal medium. The growth hormones used were IBA, IAA, NAA, 2,4-D and BAP. Stock solutions of different growth regulators were prepared at 100-1000 times concentrations. Different concentrations (0.25, 0.5, 0.75 and 1.00 mg/L) and different combinations (IAA+IBA and IBA+NAA) of growth hormones were tried for the rapid multiplication of roots. Root explants of *Cicer arietinum* L. were cultured on full strength MS medium with different hormonal concentrations of auxins (IBA, IAA, NAA & 2,4-D) and cytokinin (BAP) for upto 6 weeks for the induction of adventitious roots.

Root production in liquid MS media. Roots produced in MS medium were taken, excised into pieces and transferred into 250 mL conical flasks containing MS liquid medium. These flasks were placed in temperature-controlled gyratory shaker (Gallenkemp) at 20-25ºC for the efficient growth of roots upto six weeks under continuous shaking condition of 120 rpm.

RESULTS AND DISCUSSION

Root cultures were developed by growing seedlings of chickpea under *in vitro* conditions, taking roots as explant and growing them on MS medium. Then the comparison of different hormones was made in respect to *in vitro* rooting response of *Cicer arietinum* L. in full strength MS medium. Root explants of *Cicer arietinum* were cultured on full strength MS medium with different concentrations (0.25, 0.50, 0.75 and 1.00 mg/L) of IBA, IAA, NAA, 2,4-D and BAP.

Effects of IBA, BAP and IAA on roots induction. IBA and BAP were found to be the best among all these phytohormones used for the production of root culture. IBA in a concentration of 0.25 and 1.00 mg/L showed no initiation of roots (-) in full strength MS medium while moderate rooting response (++) was observed by 0.5 and 0.75 mg/L concentrations of IBA (Table I) (Fig. 1 & 2). Jia and Eaton (1984) observed that rooting was improved by IBA treatment in half-strength MS medium. Drew *et al*. (1993) reported that root initiation of papaya shoots was higher using IBA than IAA and NAA. Different concentrations of BAP were tried for the induction of roots in *Cicer arietinum* and it was found that BAP in concentrations of 0.25, 0.75 and 1.00 mg/L resulted in moderate root formation (++) in full strength MS medium after six weeks of culturing (Table I) (Fig. 3, 4), while very little rooting (+) was achieved with the addition of IAA after five weeks. Fernandez *et al*. (1998) germinate seeds on MS medium containing 0, 4 and 11 mg/L BAP and observed significant differences between solid and liquid media with average rooting being 16.5 and 84.7%, respectively. IAA was found to be least effective with respect to rooting. The results were in line with those of Richard *et al*. (1985) who explained that IAA was the least effective auxin in relation to rooting response.

Effects of NAA and 2,4-D on roots induction. Effects of NAA and 2,4-D on root induction were analyzed by using different concentrations of NAA and 2,4-D separately in full strength MS medium. No rooting response (-) was observed with all the concentrations of NAA and 2,4-D. Instead of rooting, an outstanding result was achieved by the

Table I. Effect of different concentrations of IBA, BAP and IAA on root culture response in *Cicer arietinum* L.

Hormonal concentrations (mg/L)	No. of flasks	Root culture response after						Rooting % (after 6 weeks)	Remarks
		Ist week	II week	III week	IV week	V week	VI week		
IBA									
0.25		-	-	-	-	-	-	-	No root formation
0.50		-	-	+	+	++	++	85	Roots were formed
0.75		-	-	+	+	++	++	90	Roots were formed
1.00		-	-	-	-	-	-	-	No root formation
IAA									
0.25		-	-	-	-	-	-	-	No root formation
0.50		-	-	-	-	-	-	-	No root formation
0.75		-	-	-	-	-	-	-	No root formation
1.00	20 (In each case)	-	-	-	-	+	+	85	Slight root initiation
BAP									
0.25		-	-	-	+	+	++	90	Roots were formed
0.50		-	-	-	-	-	-	-	No root formation
0.75		-	-	+	+	++	++	90	Roots were formed
1.00		-	-	-	-	+	++	85	Roots were formed

Basal Medium: Full Strength MS Medium. Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.

Table II. Effect of different concentrations of NAA and 2,4-D on root culture response in *Cicer arietinum* L.

Hormonal concentrations (mg/L)	No. of flasks	Callogenic response after						Rooting % (after 6 weeks)	Remarks
		Ist week	II week	III week	IV week	V week	VI week		
NAA									
0.25		-	-	-	+	++	++	85	No root formation but callus was
0.50		-	-	+	+	++	++	90	observed in all the
0.75		-	-	-	+	+	++	85	concentrations of NAA
1.00		-	-	-	+	++	++	85	
2,4-D									
0.25	20 (In each case)	-	-	-	+	++	+++	95	Instead of root formation, callus
0.50		-	-	+	++	+++	+++	95	was observed in all the
0.75		-	-	-	+	++	++	90	concentrations of 2,4-D
1.00		-	-	+	+	++	+++	95	

Basal Medium: Full Strength MS Medium. Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.

Table III. Effect of different phytohormonal combinations on the *in vitro* root culture response in *Cicer arietinum* L.

Hormonal concentrations/combinations (mg/L)	No. of flasks	Root culture response after						Rooting % (after 6 weak)	Remarks
		Ist week	II week	III week	IV week	V week	VI week		
IAA + IBA									
0.25 + 0.25		-	-	-	-	-	-	-	No root formation
0.50 + 0.50		-	-	-	+	++	++	90	Roots were formed
0.75 + 0.75		-	-	-	-	+	++	90	Roots were formed
1.00 + 1.00		-	-	-	+	++	+++	95	Roots were formed
IBA + NAA									
0.25 + 0.25		-	-	-	-	-	+	85	Slight root initiation
0.50 + 0.50	20 (in each case)	-	-	-	-	+	+	85	Roots were formed Roots
0.75 + 0.75		-	-	-	+	++	++	90	were formed
1.00 + 1.00		-	-	+	++	++	+++	95	Roots were formed

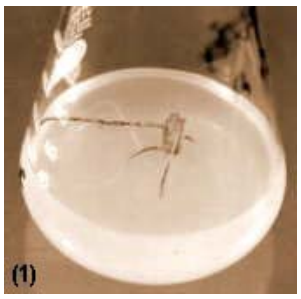
Basal Medium: Full Strength MS Medium. Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.

Table IV. Effect of half and full strength MS basal medium containing no hormone on the root culture response in *Cicer arietinum* L.

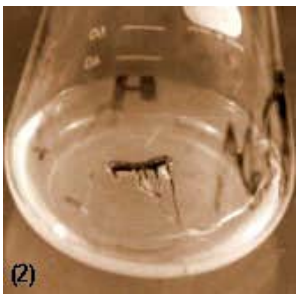
MS basal medium	No. of flasks	Root culture response after						Rooting % (after 6 weeks)	Remarks
		Ist week	II week	III week	IV week	V week	VI week		
Half strength MS medium	20	-	+	++	+++	+++	++++	95	Adventitious roots were formed
Full strength MS medium	20	-	-	+	++	++	+++	95	Adventitious roots were formed

Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.

Fig. 1-4. Effect of different concentrations of IBA and BAP on root culture response in *Cicer arietinum* L. Basal Medium: Full Strength MS Medium. Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.



Rooting response: ++
Culture period: 5 weeks
Hormonal concentration: IBA (0.5 mg/L)
General appearance: Root formation occurred.



Rooting response: ++
Culture period: 5 weeks
Hormonal concentration: IBA (0.75 mg/L)
General appearance: Root formation achieved.

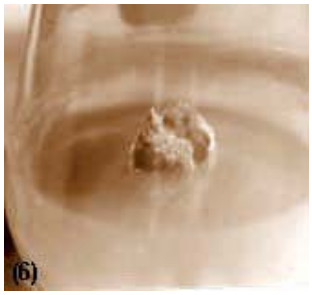
Rooting response: ++
Culture period: 6 weeks
Hormonal concentration: BAP (0.25 mg/L)
General appearance: Single Root formation.

Rooting response: ++
Culture period: 5 weeks
Hormonal concentration: BAP (0.75 mg/L)
General appearance: Root formation initiated.

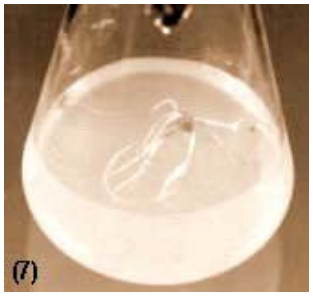
Fig. 5-6. Effect of different concentrations of NAA and 2,4-D in full strength MS medium on root culture response in *Cicer arietinum* L. Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.

Fig. 7-8. Effect of half and full strength MS basal medium containing no hormone on root culture response in *Cicer arietinum* L. Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.

Rooting response: -
Callogenic response: ++
Culture period: 5 weeks
Hormonal concentration: NAA (0.5 mg/L)
General appearance: No Root formation but compact and nodular callus with yellowish green patches was observed.



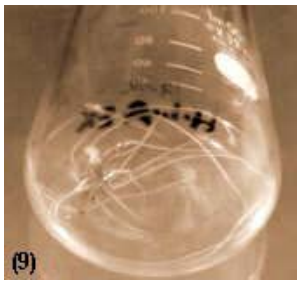
Rooting response: -
Callogenic response: +++
Culture period: 5 weeks
Hormonal concentration: 2,4-D (0.5 mg/L)
General appearance: No root formation but compact hard and nodular callus with lush green patches.



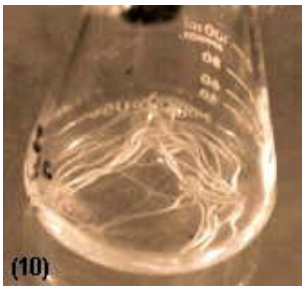
Rooting response: ++++
Culture period: 6 weeks
Hormonal concentration: No hormone added.
General appearance: Adventitious root formation.
Medium: Half strength MS basal medium.

Rooting response: +++
Culture period: 6 weeks
Hormonal concentration: No Hormone added
General appearance: Adventitious root formation.
Medium: Full strength MS basal medium.

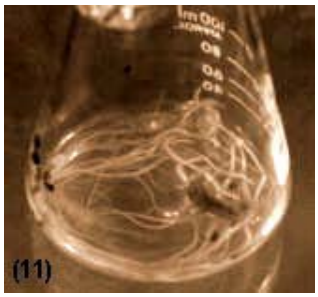
Fig. 9-12. Effect of half strength liquid MS medium containing no hormone on root culture response in *Cicer arietinum* L. Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.



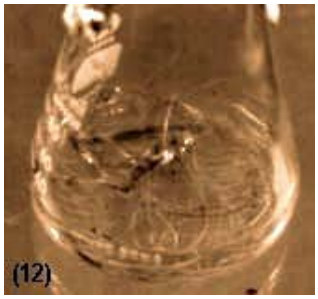
Rooting response: ++++
Culture period: 3 weeks
General appearance: Increased Root formation occurred.



Rooting response: +++++
Culture period: 4 weeks
General appearance: Efficient root formation occurred.



Rooting response: ++++++
Culture period: 5 weeks
General appearance: Excellent root formation.



Rooting response: ++++++
Culture period: 6 weeks
General appearance: Excellent root formation occurred.

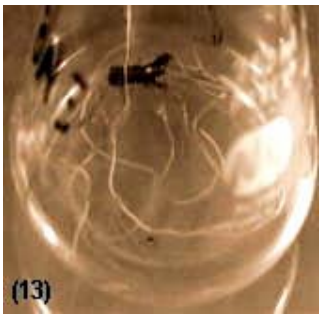
production of adequate callus (+++) in full strength MS medium when different concentrations of NAA and 2,4-D were tried for the induction of roots. Different concentrations of NAA and 2,4-D resulted in callogenic response rather than the induction of roots. Between NAA and 2,4-D, 2,4-D was found better for the induction of callus (Table II). In case of NAA, callus obtained was compact and nodular with yellowish green patches (Fig. 5) while in case of 2,4-D, compact hard, nodular with lush green patches of callus was produced (Fig. 6). The results were in line of those of Morjane and Harrabi (1993) who transferred root explants in MS medium devoid of cytokinins but containing 2,4-D and NAA for callus production. They achieved good callus production with 2,4-D. Hussey (1986) found that the most active auxin was 2,4-D, which was used to induce rapid callus proliferation.

Effect of different combinations of phytohormones on roots induction. Different combinations of phytohormones (IAA, IBA, and NAA) were tried for initiation of roots in *Cicer arietinum*. One week old root explants were inoculated on full strength MS basal medium supplemented with a combination of IAA and IBA both containing concentrations 0.25, 0.5, 0.75 and 1.0 mg/L and IBA and NAA combination both in concentrations of 0.25, 0.5, 0.75 and 1.0 mg/L. With 1.00 mg/L concentration of IAA, IBA and BAP in both IAA+IBA and IBA+NAA combinations, same, adequate rooting response (+++) was observed (Table III). Optimum rooting for winter hardy roses was achieved with high IAA and intermediate IBA and NAA concentration (Neville *et al*., 1995). The work was in line of Khosh and Sink (1982) who reported NAA plus IBA or IAA increased rooting more than IAA, IBA or NAA alone.

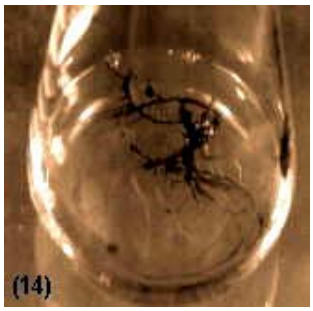
Effect of half and full strength MS medium on root induction. Effect of half and full strength MS medium containing no hormones was determined on *in vitro* initiation of roots in *Cicer arietinum* (chickpea). One week old root explants of *Cicer arietinum* L. were cultured on half and full strength MS medium containing no hormone under 18/6 light/dark cycle and at light intensity of 2000 lux. Comparatively better root production was observed on half strength MS medium containing no hormone rather than that supplemented with different concentrations and combinations of phytohormones that resulted in less root formation. Rooting response was observed after 2 week of root explant inoculation (+) and culture was continued to multiply till the end of experiment (6 week) that result in sufficient production of roots (Table IV). Good root culture (++++) was observed in half strength MS basal medium lacking of hormones after 6 weeks (Fig. 7) while full strength MS basal medium lacking hormones produce adequate root culture (+++) after 6 weeks (Fig. 8). Half strength MS medium (containing no hormone) was therefore considered better for root production than full strength MS medium (containing no hormone). The work was in line of those of Polisetty *et al.* (1996) who observed maximum rooting of chickpea when cultured in half strength MS medium. Inorganic salts in MS medium were enough to support the maximum root formation *in vitro* and the use of IBA or other auxins was not necessary (Sarwar & Flegmann, 1989).

Root cultures in solid and liquid MS media. Among solid and liquid MS media, half strength liquid MS medium was found to be best for root culturing as compared to half strength solid MS medium. There was observed significant differences between solid and liquid MS media (lacking hormones), with maximum rooting in liquid MS medium (Fernandez *et al*., 1998). Best rooting response (++++) was achieved in half strength liquid MS medium after 3 weeks of inoculation and root culture was continued to increase throughout the experiment i.e. upto 6 weeks (Fig. 9-12). Excellent rooting (++++++) was observed in half strength liquid MS basal medium lacking of hormones after 6 weeks (Fig. 12). Shaking had a great effect on multiplication of roots in liquid MS medium. Shaking increased the rate of respiration of roots and provided the roots to equal exposure to all the components of medium, which affect the increased

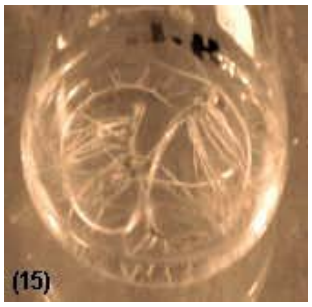
Fig. 13-16. Multiplication of roots in half strength liquid MS medium containing no hormone for maintaining root culture of *Cicer arietinum* L. Photoperiod: 18/6 L/D Cycle. Light Intensity: 2000 lux.



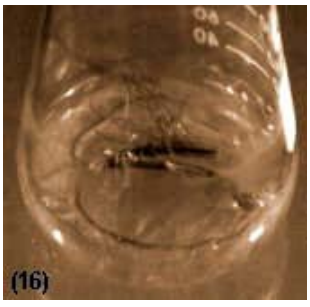
Rooting response: +++
Culture period: 4 weeks
General appearance: Root multiplication.



Rooting response: +++
Culture period: 4 weeks
General appearance: Root multiplication.



Rooting response: ++++
Culture period: 5 weeks
General appearance: Root multiplication.



Rooting response: ++++
Culture period: 5 weeks
General appearance: Root multiplication.

in growth rate. Montserrat *et al.* (1997) examined the growth in shoot-clump cultures of *Narcissus confusus* in liquid-shake medium. They explained that regenerated plants were significantly greater in MS liquid medium provided by shaking.

Finally, root culture was maintained on liquid MS medium in order to check the viability of root culture. Three months old roots (produced for 1.5 month in half strength MS basal medium and for 1.5 month in half strength MS liquid medium) were excised into pieces and again transferred to half strength MS liquid medium containing no hormone. These flasks were placed in the temperature control shaker (Gallenkemp) (120 rpm) for the efficient multiplication of roots at 20-25ºC. Bulk of root culture produced by sub-culturing the roots in half strength liquid MS medium again and again showed that root cultures were remained alive (Fig. 13-16).

CONCLUSION

The results of present investigation illustrate that the addition of different concentrations and combinations of growth hormones in the medium affect the growth of roots but their lack from the MS medium resulted in excellent root formation. Half strength liquid MS medium containing no growth hormone was found to be best for root culturing. These findings will help in proper maintenance and multiplication of root cultures to meet the continuous supply of secondary metabolites of nutritional and medicinal importance.

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(Received 18 February 2005; Accepted 20 May 2005)