Effects of Growth Regulators and Explants on Callus Induction and Organogenesis in *Hypericum perforatum*

I. Sharifi, M.H. Fotokian, A. Kordenaeij and S. Ramazani
College of Agriculture and Medicinal Plant Research Center, Shahed University
Tehran
Iran

T. Hasanlu, S.M. Khayyam Nikoei, D. Davoodi and B. Nakhoda
Agricultural Biotechnology Research Institute
Karaj
Iran

G. Mohammadinejad
College of Agriculture
Shahid Bahonar University
Kerman
Iran

Keywords: BAP, IAA, MS medium, root induction, shoot induction

Abstract

***Hypericum perforatum* is a traditional medicinal plant that has been used for the treatment of neurological disorders and depression. In this research, the effect of different levels of growth regulators (IAA and BAP) and explants type (root, stem, leaf) on callus induction and organogenesis of *Hypericum perforatum* were studied through factorial experiment design based on completely randomized arrangement with five replications and five samples per experimental unit. Explants from sterilized seedlings were cultured on MS medium containing different concentrations of IAA (0.0, 0.5, 1.0, and 2.0 mg L^{-1}) and BAP (0.0, 0.4, and 0.8 mg L^{-1}). We incubated the samples in the dark at 23°C for 28 days. After this period, variables including callus size, number of roots and number of shoots were measured. Among tested explants, leaf explants was found to be most effective as it produced the most number of roots and shoots. The main effects of IAA and BAP and also the interaction or combination effects of these growth regulators were statistically significant ($P \leq 0.01$) on all traits. The maximum number of shoots was obtained in leaf explants, when the concentration of BAP and IAA was high and low, respectively. Our findings are compatible with the results reported that in *H. perforatum*, BAP was found to be the most efficient in promoting shoot regeneration when leaves were used as the explants.**

INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. In-vitro regeneration holds tremendous potential for the production of high-quality plant-based medicine.

St. John's wort (*Hypericum perforatum* L.) is a well-known traditional medicinal plant that has been used for centuries for the treatment of several diseases, such as skin lesions, eczema, cancer, burns and microbial, inflammatory, anti-oxidant and psychological disorders (Dias et al., 1998; Ishiguro et al., 1998; Barnes et al., 2001; Sanchez-Mateo et al., 2002; Agostinis et al., 2002; Silva et al., 2005).

In vitro systems have been reported as an effective tool for obtaining genetically uniform plants, which can be a source of variable pharmaceutical preparations (Santarem and Astarita, 2003). Plant regeneration of the *Hypericum* species has been achieved by using as explants the whole seedling or their excised parts (Cellarova et al., 1992; Bernardi et al., 2007; Ayan and Cirak, 2008; Namli et al., 2009; Namli et al., 2010), hypocotyl sections (Murch et al., 2002; Zobayed et al., 2004), leaves (Pretto and Santarem, 2000), leaf discs and stem segments (Ayan et al., 2005), and adventitious roots

(Goels et al., 2009) using various types and concentration of cytokinins and auxin.

The aim of this research was to evaluate the effects of different explants and plant growth regulators on callus induction, roots and shoots production in *H. perforatum* L. Investigation of hypericine content in callus regenerated through suspension culture is however intended in the near future.

MATERIALS AND METHODS

Seeds of *Hypericum perforatum* L. were obtained from Medicinal Plant Research Center, Shahed University, Tehran, Iran. They were washed in running tap water and were surface-sterilized by immersing in a 5% (w/v) commercial bleach solution (NaOCl) for 20 minutes. Subsequently, they were washed 3 times with sterilized distilled water for 12 minutes Surface-sterilized seeds were placed on hormone-free MS (Murashige and Skoog, 1962) basal medium supplemented with 3% Sucrose and 0.8% Agar for germination. All chemicals were purchased from Sigma. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 15 min. Cultures were incubated at 25±2°C under 16 hours of photoperiod from cool white fluorescent tube giving 2400 lux at culture level. Callus cultures were initiated using different explants (root, stem, leaf) from a 40-day-old plantlet grown in vitro. The effect of growth regulators was tested using different combinations of Indole-3-acetic acid (IAA, 0.5, 1.0 and 2.0 mg L^{-1}) and N6-benzylaminopurine (BAP, 0.4 and 0.8 mg L^{-1}). Medium without plant growth regulators was used as a control. Cultures were kept in darkness at 23°C for 4 weeks without sub-culturing. Five explants were cultured per Petri plate (1.8×6.0 cm) sealed with parafilm, in 5 replicates, representing a total of 25 observations per treatment.

The study was performed as a whole, one time and data recorded (means of callus size, number of induced shoots, and frequency of rooting) were analyzed using ANOVA. Experimental design was factorial based on completely randomized. Statistically significant averages were compared using Duncan's multiple range test. The statistical analysis was performed using SPSS software ver. 16.

RESULTS AND DISCUSSION

The explant type (root, stem, leaf) had a significant effect on the regeneration of shoots, roots and callus size (Table 1). Similar variation in the regeneration frequency of shoots based on explant types was also reported previously in *H. perforatum* (Zobayed and Saxena, 2003). The plant growth regulator combination for optimal regeneration in *H. perforatum* varied with the explant type in the present study as reported in other species (Nikam and Shitole, 1999; Dhar and Joshi, 2005). In the results reported here for *H. perforatum*, the regeneration response is clearly a plant growth regulator-driven explant-dependent phenomenon.

Among the explant types, leaf segments quickly responded to the media and produced the highest frequency of callus, root and shoot buds. Among tested explants, leaf explants were found to be most effective as they produced the maximum number of roots and shoots (Table 2).

Effect of Plant Growth Regulator

The main effects of IAA and BAP and also the interaction or combination effects of these growth regulators were statistically significant ($p \leq 0.01$) on callus size, number of roots and number of shoots, regardless of explants type (Table 1).

In this study, increasing concentrations of IAA reduced the number of shoots. Similar responses were observed in BAP concentrations for callus size and number of roots. Our findings are compatible with those of Pretto and Santarem (2000), who reported that in *H. perforatum*, BAP was found to be the most efficient in promoting shoot regeneration when leaves were used as the explants. The concentration of 0.2 mg L^{-1} of IAA proved to be the most effective for callus size and number of roots. Low concentrations of IAA (0.0, 0.5 and 1.0 mg L^{-1}) have not been effective on callus formation and number of roots. The differences between BAP concentrations (0.4 and 0.8

mg L^{-1}) were not statistically significant when the explants were root and leaf. The maximum number of shoots was obtained in leaf explants, when the concentration of BAP and IAA was high and low, respectively (Fig. 1).

As a result, the present investigation showed that adventitious shoots could be regenerated directly from explants (root, stem, leaf) of *H. perforatum* L. The increase in IAA concentration (1.0 and 2.0 mg L^{-1}) resulted in significant increase in the number of roots. Shoot regeneration was higher in medium containing high concentrations of BAP either with or without IAA. In contrast, high levels of IAA (0.1 and 0.2 mg L^{-1}) delayed the production of shoot buds and often resulted in the formation of callus irrespective of explant.

Callus Induction and Organogenesis

In our findings, the highest size of induced callus was obtained from the leaf discs cultivated on the medium supplemented with 2.0 mg L^{-1} of IAA (without BAP) (Fig. 1). According to previous reports, 2,4-D and kinetin proved to be effective plant growth regulators for callus induction and cell suspension cultures from *H. perforatum* L. (Bais et al., 2002; Travis et al., 2002).

The highest number of root (3.52 roots/explant) was obtained from leaf segments cultured in the medium containing 2.0 mg L^{-1} of IAA (without BAP). This was followed by stem discs cultured in the same medium with 2.88 roots/explant.

In vitro shoot formation may be subjected to change depending upon the explant types used (Zobayed and Saxena, 2003). Likewise, in this study the average number of shoots was significantly higher in calli obtained from leaf discs than in those from stem and root segments (Table 2). The calli obtained from leaf discs and cultured on the medium supplemented with 0.4 and/or 0.8 mg L^{-1} of BAP (without IAA) had the highest shoot formation (3.32 shoots/explant), followed by the calli obtained from leaf discs and cultured on the medium supplemented with 1 mg L^{-1} of IAA, and 0.4 mg L^{-1} of BAP (3.08 shoots/sample). Studies concerning shoot induction and multiplication in *H. perforatum* L. have reported an average 20-22 shoots, depending on the factors evaluated (Bezo and Stefunova, 2001; Pretto and Santarem, 2000). However, it is important to note that induction of up to 50 shoots per callus for *H. perforatum* L. was reported by Santarem and Astarita (2003) employing thidiazuron and NAA in addition to BA and kinetin as callus inducing agent. The marked difference between the 2 reports may derive from the plant growth regulators used.

The effect of IAA on root induction was found to be highest (3.52 roots/leaf explant) in 2.0 mg L^{-1} of IAA (without BAP).

CONCLUSION

Hypericum genus is commonly used as a medicinal plant for its anti-depressant properties. Harvesting such medicinal plants from nature is causing a loss of genetic diversity. Plants with very small seeds like *Hypericum* cannot be cultured by traditional methods, easily. The plant has many active compounds (for example, hypericine) and is traditionally used for its sedative, antihelminthic, anti-inflammatory and antiseptic effects. It is also reported to be effective in the treatment of burns and in the treatment of gastrointestinal diseases (Conforti et al., 2002). Directly adventitious shoots formation without callus phase is very important. Plants produced by direct organogenesis may exhibit greater genetic stability than those produced from callus (Lee and Phillips, 1988; Karam and Al-Majathoub, 2000). The effects of auxins and cytokinins on shoot multiplication have been reported earlier for *Hypericum* species (Cellarova et al., 1992; Moura, 1998). This study describes the procedures for adventitious shoot induction from different explants (root, stem and leaf) of *H. perforatum* and different combined concentrations of IAA and BAP.

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as in-vitro regeneration and genetic transformations. It can also be harnessed for production of secondary metabolites using

plants as bioreactors. This paper reviews the achievements and advances in the application of tissue culture for the in-vitro regeneration of medicinal plants from various explants. A simple, efficient, high frequency protocol on direct regeneration from root, stem and leaf explants of *H. perforatum* has been demonstrated here using combined concentrations of IAA and BAP. Additionally, this method could be useful for large-scale multiplication as well as in vitro conservation of germplasm of this medicinal species.

Callus induction and proliferation are useful tools for the study of biosynthesis of natural products and the factors affecting it (Pretto and Santarem, 2000) and plant cell suspension cultures are often an effective system in which to study the biological significance of bioactive metabolites under in vitro conditions, as well as for producing natural products for bioprocessing applications (Travis et al., 2002). The in vitro production of hypericin (Bais et al., 2002), pseudohypericin (Kirakosyan et al., 2001), hyperforin (Sirvent and Gibson, 2002) and flavonoids (Kartnig and Brantner, 1990) has been reported previously. To our knowledge, there is no report on the induction of callus, root and shoot nor the indirect regeneration in *H. perforatum*. Due to the potential for adding value to *H. perforatum*, in vitro regeneration of plants has been investigated as an option for multiplication of elite plants and production of valuable phytopharmaceuticals.

ACKNOWLEDGEMENTS

This work was supported by Shahed University and Agricultural Biotechnology Research Institute of Iran (ABRII).

Literature Cited

Agostinis, P., Vantieghem, A., Merlevede, W. and Peter, A.M. 2002. Hypericin in cancer treatment: More light on the way. Int. J. Biochem. Cell Biol. 34:221-241.

Ayan, A.K., Çirak, C., Kevseroglu, K. and Sokmen, A. 2005. Effects of explant types and different concentrations of sucrose and phytohormones on plant regeneration and hypericin content in *Hypericum perforatum* L. Turk. J. Agric. For. 29:197-204.

Ayan, A.K. and Çirak, C. 2008. Variation of hypericins in *Hypericum triquetrifolium* Turra growing in different locations of Turkey during plant growth. Nat. Prod. Res. 22(18):1597-1604.

Bais, H.P., Walker, T.S., McGrew, J.J. and Vivanco, J.M. 2002. Factors affecting growth of suspension culture of *Hypericum perforatum* L. and production of hypericin. In vitro Cell Develop. Biol. Plant 38:58-65.

Barnes, J., Anderson, L.A. and Phillipson, J.D. 2001. St John's wort (*Hypericum perforatum* L.): review of its chemistry, pharmacology and clinical properties. J. Pharm. and Pharmacol. 53:583-600.

Bernardi, A., Maurmann, N., Rech, S. and Poser, G. 2007. Benzopyrans in *Hypericum polyanthemum* Klotzsch ex Reichardt cultured in vitro. Acta Physiol. Plant 29(2):165-170.

Bezo, M. and Stefunova, V. 2001. Indirect regeneration of *Hypericum perforatum* L. under in vitro conditions. Acta Fytotechnica et Zootechnica 4:277-279.

Cellarova, E., Kimakova, K. and Brutovska, R. 1992. Multiple shoots formation in *Hypericum perforatum* L. and variability of R0. Theor. Appl. Genet. 101:46-50.

Conforti, F., Statti, GA., Tundis, R., Menichini, F. and Houghton, P. 2002. Antioxidant activity of methanolic extract of *Hypericum triquetrifolium* Turra aerial part. Fitoterapia 6:479-483.

Dhar, U. and Joshi, M. 2005. Efficient plant regeneration protocol through callus for *Saussurea obvallata* (DC.) Edgew. (*Asteraceae*): effect of explant type, age and plant growth regulators. Plant Cell Rep. 24:195-200.

Dias, A.C.P., Francisco, A., Barberan, T., Ferreria, F.M. and Ferreres, F. 1998. Unusual flavanoids produced by callus of *Hypericum perforatum* L. Phytochem. 48:1165-1168.

Goel, M.K., Kukreja, A.K. and Bisht, NS. 2009. In vitro manipulations in St. John's wort (*Hypericum perforatum* L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD

analysis. Plant Cell Tissue Organ Cult. 96:1-9.
Ishiguro, K., Nagareya, N. and Fukomoto, H. 1998. A phloroglucinol derivative from cell suspension cultures of *Hypericum perforatum* L. Phytochem. 47:347-369.
Karam, N.S. and Al-Majathoub, M. 2000. Direct shoot regeneration and microtuberization in wild *Cyclamen persicum* Mill. using seedling tissue. SciHort. 86:235-246.
Kartnig, T. and Brantner, A. 1990. Secondary constituents in cell cultures of *Hypericum perforatum* and *Hypericum maculatum*. Planta Med. 56:634-637.
Kirakosyan, A., Hayashi, H., Inoue, K., Charchoglyan, A., Vardapetyan, H. and Yammoto, H. 2001. The effect of cork pieces on pseudohypericin production in cells of *Hypericum perforatum* L. shoots. Russian J. Plant Physiol. 48:816-819.
Lee, M. and Phillips, RL. 1988. The chromosomal basis of somaclonal variation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39:413-437.
Moura, M. 1998. Conservation of *Hypericum foliosum* Aiton, an endemic azorean species, by micropropagation. In vitro Cellular Dev. Biol. Plant. 34:244-248.
Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473-479.
Murch, S.J., Rupasinghe, H.P.V. and Saxena, P.K. 2002. An in vitro and hydroponic growing system for hypericin, pseudohypericin, and hyperforin production of St. John's wort (*Hypericum perforatum* cv New Stem). Planta Medica 68:1108-1112.
Namli, S., Toker, Z., Isikalan, C. and Ozen, H.C. 2009. Effect of UV-C on production of hypericin in *H. triquetrifolium* Turra grown under in vitro conditions. Fresenius Environ. Bull. 18(1):123-128.
Namli, S., Akbas, F., Isikalan, C., Tilkat Ayaz, E. and Basaran, D. 2010. The effect of different plant hormones (PGRs) on multiple shoots of *Hypericum retusum* Aucher. P.O.J. 3(1):12-17.
Nikam, T.D. and Shitole, M.G. 1999. In vitro culture of Safflower L. cv. Bhima, initiation, growth and growth optimization and organogenesis. Plant Cell Tiss. Organ Cult. 55:15-22.
Pretto, F.R. and Santarem, E.R. 2000. Callus formation and plant regeneration from *Hypericum perforatum* leaves. Plant Cell Tiss. Organ Cult. 62:107-113.
Sanchez-Mateo, C.C., Prado, B. and Rabanal, R.M. 2002. Antidepressant effects of the methanol extract of several Hypericum species from the Canary Islands. J. Ethnopharmacol. 79:119-127.
Santarem, E.R. and Astarita, L.V. 2003. Multiple shoots formation in *Hypericum perforatum* L. and hypericin production. Braz. J. Plant Physiol. 15:43-47.
Silva, B.A., Ferreres, F., Malva, J.O. and Dias, A.C.P. 2005. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. Food Chem. 90:157-167.
Sirvent, T. and Gibson, D. 2002. Induction of hypericins and hyperforin in *Hypericum perforatum* L. in response to biotic and chemical elicitors. Physiol. Mol. Plant Path. 60:311-320.
Travis, S., Bais, W. and Vivanco, H.P. 2002. Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L. (St. John's wort). Phytochem. 60:289-293.
Zobayed, S.M.A., Murch, S.J., Rupasinghe, H.P.V. and Saxena, P.K. 2004. *In vitro* production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'). Plant Sci. 166:333-340.
Zobayed, S.M.A. and Saxena, P.K. 2003. In vitro-Grown Roots: A Superior explant for prolific shoot regeneration of St. John's wort (*Hypericum perforatum* L. 'New Stem'). In a Temporary Immersion Bioreactor. Plant Sci. 165:463-470.

Tables

Table 1. Results of variance analysis for traits under study.

Source of variation	Degree freedom	Mean of square								
		Root explant			Stem explant			Leaf explant		
		Callus size	No. root	No. shoot	Callus size	No. root	No. shoot	Callus size	No. root	No. shoot
IAA	2	10.1**[1]	9.8**	86.2**	15.7**	13**	74.7**	15.2**	19.2**	67.2**
BAP	2	3.6**	63.5**	9.1**	2.8**	46.4**	43.4**	11.4**	144**	89.6**
IAA*BAP	6	8.6**	9.86**	39**	7.3**	6.7**	35.4**	26.6**	19.2**	35.4**
Error	48	0.07	0.63	0.85	0.26	3.18	1.13	0.32	0.14	1.01
Sampling error	240	0.25	0.052	0.92	0.51	0.04	1.36	0.17	0.21	0.92

**Significant at the 1% level of probability.

Table 2. Results of mean comparisons among main effects of IAA and BAP on callus size, number of roots and number of shoots in different explants.

PGR	Root explant			Stem explant			Leaf explant		
	Callus size (mm)	No. of root	No. of shoot	Callus size (mm)	No. of root	No. of shoot	Callus size (mm)	No. of root	No. of shoot
IAA									
0.0	1.5a[1]	0.5a	3.44c	0.5a	0.5a	3.5c	0.5a	0.5a	3.63c
0.5	1.5a	0.92b	1.9b	0.5a	1.02b	2.27b	0.5a	1.16b	2.46b
1.0	1.67b	1.03c	1.2a	1.02b	1.27c	1.34a	1.26b	1.43c	1.66a
2.0	1.22c	1.38d	1.14a	1.44c	1.46d	1.44a	1.3b	1.67d	1.6a
BAP									
0.0	0.56a	1.88a	1.59a	0.71a	0.5a	1.38a	0.5a	2.58b	1.25a
0.4	0.86b	0.5a	2b	0.85a	0.87b	2.5b	1.1b	0.5a	2.81b
0.8	0.96b	0.5a	2.18b	1.04b	1.82c	2.54b	1.07b	0.5a	2.96b

[1]Different letters in each column show statistically significant different.

Figures

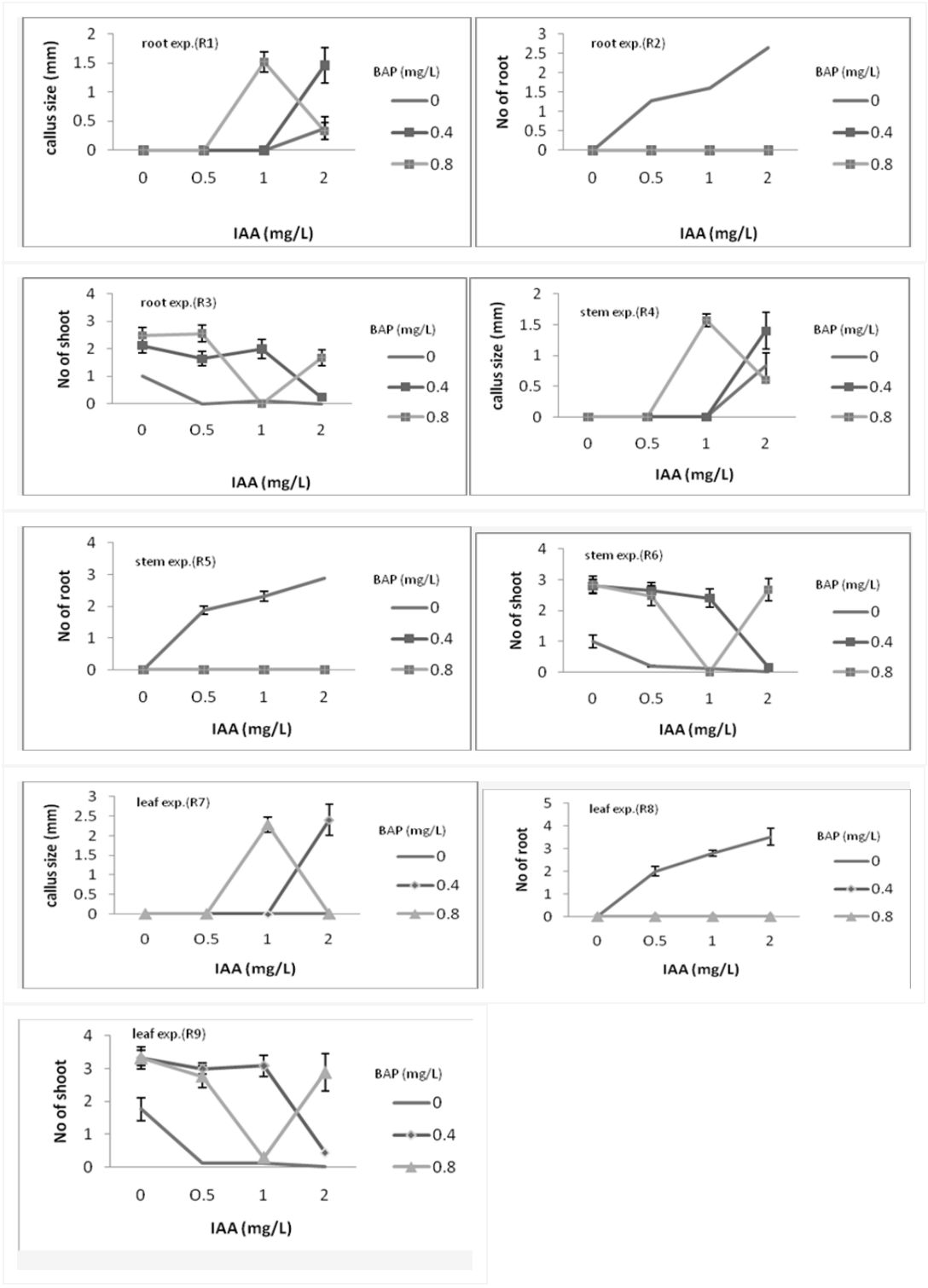

Fig. 1. The results of interaction between plant growth regulators (IAA and BAP) with explant types (root, stem, leaf) on callus size, number of root and number of shoot.