http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (21): 3900-3904, 2007
ISSN 1028-8880

Influence of Auxins Combinations on Accumulation of Reserpine in the Callus of *Rauvolfia tetraphylla* L.

[1]S. Anitha and [2]B.D. Ranjitha Kumari
[1]Department of Biotechnology, J.J. College of Arts and Science,
Pudukkottai-622 404, Tamil Nadu, India
[2]Stress Physiology and Medicinal Plant Biotechnology Unit,
Department of Plant Science, School of Life Sciences,
Bharathidasan University, Tiruchirappalli-620 024, Tamil Nadu, India

Abstract: Reserpine is a monoterpene indole alkaloid used to treat hypertension because of its hypotensive property and psychiatric disorders because of its tranquilizing effect. Protocol has been standardized to enhance the synthesis of reserpine in leaf derived calli of *Rauvolfia tetraphylla* L. by adjusting the auxins combinations in the medium consisting of MS nutrient salts and B_5 vitamins. Auxins such as naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were used in 1-5 μM concentration along with 9 μM concentration of 2,4 dichlorophenoxy acetic acid (2,4-D), which was found suitable for callus induction. The combination of (2,4-D) with NAA had been proved to accumulate maximum amount of reserpine followed by 2,4-D with IBA. The IAA with 2,4-D combination yielded very less amount of reserpine than the other combinations and 9 μM 2,4-D alone. The results suggest that there may be synergetic effect of NAA with 2,4-D and IBA with 2,4-D for increase in the biomass and reserpine accumulation and antagonistic effect of IAA with 2,4-D for the above said factors in the callus.

Key words: *Rauvolfia tetraphylla* L., callus, 2,4-D, NAA, IAA, IBA, reserpine

INTRODUCTION

Rauvolfia tetraphylla L. is a medicinally important woody shrub belongs to the family Apocynaceae. The wild population of *Rauvolfia tetraphylla* L. is endangered in India (Swarup and Arora, 2000; Faisal and Anis, 2002) due to uprooting of the plants for drug preparation. The alkaloids present in the roots of *R. tetraphylla* are reserpine, ajmalicine, ajmaline, serpentine, rescinnamine, yohimbine and tetraphyllicine (Faisal and Anis, 2002). In *Rauvolfia serpentina* (L.) Benth. ex. Kurz., a most extensively studied and highly exploited species, the total alkaloid content was reported to vary from 0.7-3.0% of total root dry mass (Dhiman, 2006) and the reserpine content was estimated as 0.1% of dry root (Anonymous, 1998). Isolated reserpine is used in the modern medicine to treat hypertension and psychological disorders, which is found to be more potent than the crude drug prepared from the root (Pullaiah, 2002) and reserpine is the first natural tranquilizer. The major problem in extracting the active principle from the field grown plants is the variation in the compound yield, both qualitatively and quantitatively, depend on specific seasons during which plant collection is performed (Rocha *et al*., 2005; Ralphs and Gardner, 2003), besides the effect of environmental factors. The literature availability on *R. tetraphylla* secondary metabolism from the field grown plants was very scanty and no research was reported on *in vitro* production of compounds except the attempts made by our group (Anitha and Ranjitha Kumari, 2006a, b). In this communication we report the production of reserpine by adjusting the auxins combinations in the growth medium.

MATERIALS AND METHODS

Callus induction: Leaf explants were collected from the field grown plants of Medicinal plants garden, Bharathidasan University, Tiruchirappalli, Tamil Nadu in the month of December 2005 and thoroughly washed in running tap water to remove the dust particles from the surface of the explants. For the surface sterilization of leaf explants and for callus induction the already explained procedure was followed (Anitha and Ranjitha Kumari,

Corresponding Author: B.D. Ranjitha Kumari, Stress Physiology and Medicinal Plant Biotechnology Unit, Department of Plant Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli-620 024, Tamil Nadu, India

2006a, b). The surface cleaned explants were taken to the laminar airflow chamber and surface sterilized with 0.1% mercuric chloride (W/V) for four minutes followed by washing with sterile distilled water for 4-5 times. The callus induction was done in MS medium (Murashige and Skoog, 1962) that was modified by supplementing with 9.04 μM 2,4-D and 40 g L^{-1} sucrose. The modification also included additions of 100 mg L^{-1} myoinositol, 2 mg L^{-1} thiamine HCl, 0.1 mg L^{-1} of riboflavin, biotin and folic acid. Auxins such as IAA, IBA and NAA ranging from 1-5 μM concentrations was added in the MS medium. The pH of the medium was adjusted to 5.7 and the medium was sterilized at 15 lb for 15 min. The sterile explants were inoculated on callus induction medium and cultures were exposed to 25±2°C and 16/8 h light/dark condition by using cool white fluorescent tubes (40 μM m^{-2} sec^{-1}). The explants were transferred to fresh medium for every 30 days. The results were recorded periodically and the data were subjected to statistical analysis.

Biomass analysis: Moisture content of the callus was analyzed all through the ten subcultures of the calli in the respective treatment medium. For the analysis, the callus was cut in to approximately 50 mg pieces and inoculated on callus induction medium in control and treatment conditions. After 30 days of culture, the calli were collected, washed with distilled water, air dried and the weight was recorded as fresh weight. The calli were dried at 40°C for 24 h and the dry weight was recorded. The percentage of moisture content of the callus was calculated by using the fresh weight and dry weight of the callus.

Alkaloid extraction and analysis: Sheludko *et al.* (1998) method was followed for extraction and analysis of alkaloids with some modifications. One gram dried callus was powdered and extracted with 80% methanol in distilled water. The extracted sample was acidified with 0.1 N HCl and neutralized with diluted NH_4OH (1%). The sample was decanted and then evaporated under vacuum and the weight was recorded as crude alkaloid content. Crude alkaloid was dissolved in a few drops of ethanol and subjected to TLC and reverse phase HPLC analysis by using reserpine (SRL products, India) as standard. Reserpine content in the crude extract was calculated and tabulated.

Statistical analysis: For each treatment 15 replicates (explants) were used and the experiments were repeated upto ten subculture period. The mean values derived from all the experiments was subjected to One way ANOVA and DMRT (Duncan's Multiple Range Test) by using SPSS software (Statistical Package for the Social Sciences) version 10.0 (LEAD Technologies Inc., Chicago, USA). The significance of the experiments was calculated at 0.05 and 0.02 significant level. The alkaloid content was analysed only in the calli which reached appreciable weight in each treatment during the ten subculture period and the mean values was presented here.

RESULTS

Callus induction: Callus induction was high in the combinational treatment of 2,4-D and NAA. The combination of IBA with 2,4-D also enhanced the callusing response. Callus induction was started from the margins and wounded regions of the leaf explants after ten days to three weeks of inoculation. In the control (2,4-D alone) callus, callusing response started after two weeks of culture whereas in NAA combinations the response was preceded three to five days. The maximum callus induction was observed as 96% in 3 μM NAA treatment. The least callusing response was observed in IAA combinations with 2,4-D and the response was 40% in 4 μM IAA treatment (Fig. 1).

Biomass analysis: There was an increase in fresh biomass observed in the NAA combination treatments at 1, 2 and 3 μM concentrations than the control and other treatments. In the control (0.3354 g) and IBA treatments (0.3400 g at 2 μM IBA) also the increase in biomass was high than the combinations of IAA with 2,4-D (Fig. 2). The similar pattern of results was observed in the dry weight of the calli also. The maximum dry weight of the

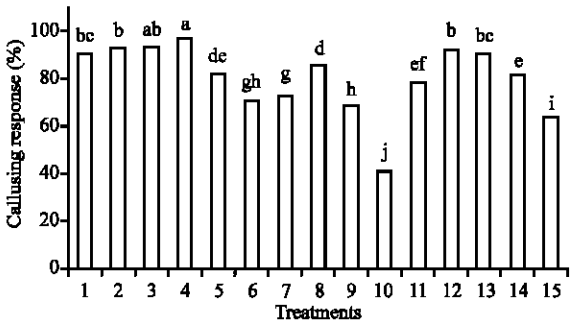

Fig. 1: Effect of different auxins combinations on callus induction in *Rauvolfia tetraphylla* L. Callusing response with, (1) - 9 μM 2,4-D (Control), (2-6) - C + 1-5 μM NAA, (7-10) - C + 1-4 μM IAA, (11-15) - C + 1-5 μM IBA treatments. According to DMRT, the alphabets followed by different letter are statistically significant at 0.02 level

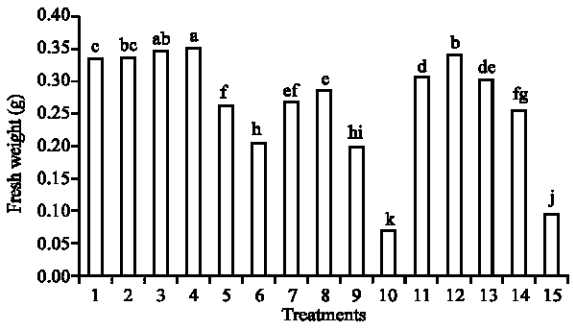

Fig. 2: Biomass increase in the callus of *Rauvolfia tetraphylla* L. grown in medium fortified with different combinations of auxins. Fresh weight in, (1) - 9 μM 2,4-D (Control), (2-6) - C + 1-5 μM NAA, (7-10) - C + 1-4 μM IAA, (11-15) - C + 1-5 μM IBA treatments. According to DMRT, the alphabets followed by different letter are statistically significant at 0.02 level

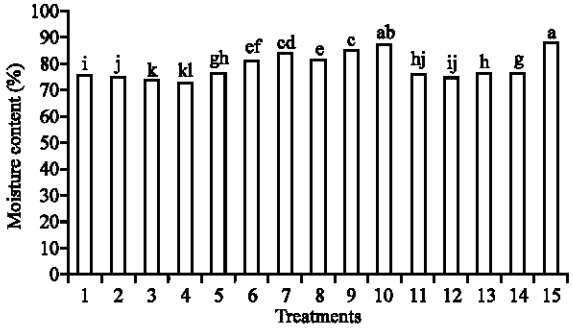

Fig. 4: Moisture content (%) in the callus of *Rauvolfia tetraphylla* L. grown in medium fortified with different combinations of auxins. Moisture content in, (1) - 9 μM 2,4-D (Control), (2-6) – C + 1-5 μM NAA, (7-10) - C + 1-4 μM IAA, (11-15) - C + 1-5 μM IBA treatments. According to DMRT, the alphabets followed by different letter are statistically significant at 0.02 level

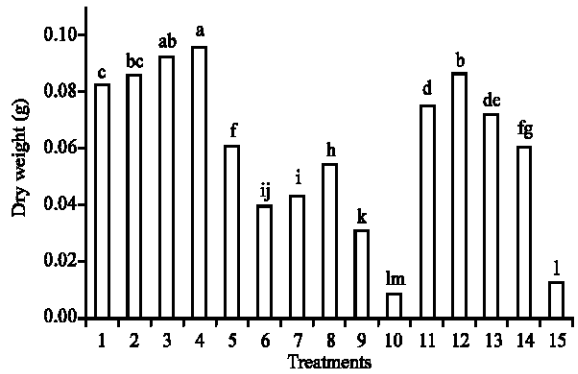

Fig. 3: Dry weight in the callus of *Rauvolfia tetraphylla* L. grown in medium fortified with different combinations of auxins. Dry weight in, (1) - 9 μM 2,4-D (Control), (2-6) - C + 1-5 μM NAA, (7-10) - C + 1-4 μM IAA, (11-15) - C + 1-5 μM IBA treatments. According to DMRT, the alphabets followed by different letter are statistically significant at 0.02 level

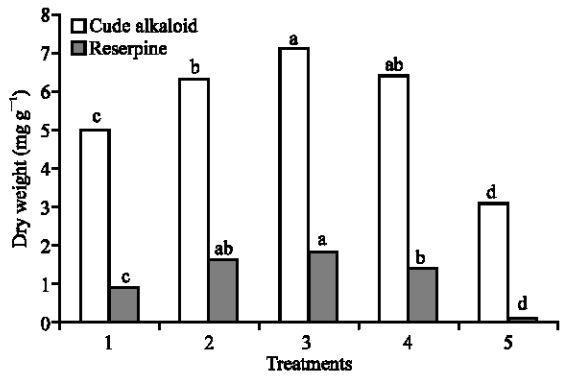

Fig. 5: Crude alkaloid and reserpine content in the callus of *Rauvolfia tetraphylla* L. grown in medium fortified with different combinations of auxins. Crude alkaloid and reserpine in (1) - 9 μM 2,4-D (Control), (2) - C + 2 μM NAA, (3) - C + 3 μM NAA (4) - C + 2 μM IBA, (5) - C + 2 μM IAA. According to DMRT, the alphabets followed by different letter are statistically significant at 0.05 level

calli was observed after drying in the oven for 24 h at 40°C in 2 μM NAA concentration as 0.0923 g and at 3 μM NAA as 0.0961 g. In 2 μM IBA treatment the dry weight was high (0.0862 g) than the calli grown in control (0.0826 g) (Fig. 3). The moisture content of the callus decreased sequentially with the increase in NAA concentration up to 3 μM NAA and then increased in 4 and 5 μM NAA treatments. Higher percentage of moisture content was observed in IAA treated calli than in all other treatments (Fig. 4).

Effect of various treatments on alkaloid accumulation in the calli: The reserpine production was stably maintained in the calli subjected to different auxins combinational treatments during the ten subculturing period. In the control callus the reserpine content was calculated as 0.9 mg g^{-1} dry weight (D. wt.). In the combined treatment with two auxins, maximum reserpine production was detected as 1.81 mg g^{-1} D. wt. in the callus treated with 3 μM NAA followed by 1.64 mg g^{-1} D. Wt. at 2 μM NAA treatment. Next maximal response was observed with 2 μM

IBA treatment as 1.51 mg g^{-1} D. wt. The reserpine content was less in the IAA combination as 0.11 mg g^{-1} D. wt. (at 2 μM IAA treatment) which was very less than the control value (0.9 mg g^{-1} D. wt.) (Fig. 5).

DISCUSSION

In general callus and cell cultures were believed to yield very less amount of secondary metabolites and various reasons had been proposed to support this view. Lindsey (1988) reported that secondary compounds are produced in specific tissue types in the plants and often only when the plant is subjected to stress or elicitation. Similarly, Charlwood *et al.* (1995) stated that as because disorganized cultures (callus, suspension and protoplast etc.) lack differentiated cell types, they are often unable to synthesize tissue-specific compounds and may also lack the ability to accumulate the secondary metabolite in the vacuole or extracellularly. A further problem with disorgamized cultures was stated that they are notoriously genetically unstable (Holden *et al*., 1988) so that they can not be used for commercial scale-up by using stable cell lines (Rhodes *et al*., 1988; Iwase *et al*., 2005). Contrary to their statements, we observed increased synthesis of reserpine in callus than the *in vivo* leaf which was used as explant in the present experiment. The reserpine content of the callus in each subculture was analyzed and we observed a stable production of reserpine in all through the period. The results we presented here for moisture content and alkaloid content was similar during all the ten subcultures of the callus in respective treatments.

The combination of two auxins resulted in more secondary metabolite accumulation and 2,4-D was necessary for initial callus induction. Baskar Rajan (2001) also reported that 2,4-D was found to be the best regulator for the callus induction and production of the alkaloid solasodine in *Solanum eleagnifolium.* Similarly 2,4-D enhanced the production of steroid diosgenin in the cultures of *Dioscorea doryophora* (Yeh *et al*., 1994). Contrarily, in *Aspidosperma ramiflorum*, the indole alkaloids production was detected in the callus but the compound concentration was less than intact plant (Olivera *et al*., 2001). Consistent with present research, production of azadirachtin and nimbin has been shown to be higher in cultured shoots and roots of *Azardirachta indica* compared to that of field grown plants (Srividhya *et al*., 1998). Narasimhan and Nair (2004) reported that in the medium supplemented with 2 mg L^{-1} 2,4-D and 0.4 mg L^{-1} BAP, production of an isoquinoline alkaloid berberine was 57.37% of total dry weight and when 2,4-D was replaced by 4 mg L^{-1} NAA, the production of berberine increased to 76.25%. Contrary to the reports supporting the enhanced effect of phytohormones on the production of secondary metabolites, Siah and Doran (1991) reported that without exogenous hormones, maximum codeine and morphine production was observed in cultures of *Papaver somniferum* and this amount is three times higher than in cultures supplied with hormones.

Radwanski and Last (1995) and Seth and Mathur (2005) reported that tryptophan is one of the least abundant amino acids present in plants and is the sole donor of indole ring for the synthesis of auxins, glucosinolates, nicotinic acid, phytoalexins and alkaloids. Hence the production of auxins and alkaloids are interlinked that they need the common precursor for the synthesis, supply of auxins (NAA and IBA) in the culture medium may enhanced the alkaloid synthesis. Moreover the indole ring of the supplemented hormones may also play a role in alkaloid synthesis. In the case of IAA, the reason for reduced alkaloid production is not clear, there may be a chance for the degradation of this auxin during autoclaving process and or it may interfered with the activity of 2,4-D. In our previous investigation, we reported enhanced synthesis of reserpine in the calli when they fed with tryptophan (Anitha and Ranjitha Kumari, 2006a), a precursor of alkaloids and auxins. However, the biomass increase was very less in the calli fed with tryptophan. In the present study we found the combinational auxins supplementation induced more biomass as well as reserpine accumulation in the callus, this may be a promising approach for the production of reserpine at industry level.

In conclusion, synthesis and accumulation of reserpine in cultured cells of *Rauvolfia tetraphylla* L. provide an opportunity for bypassing the plant because the plant root contains the active principle and hence in the conservation of this species which is endangered. A culture system with combination of two hormones (2,4-D and NAA) is desirable for the enhanced production of reserpine where the calli were dark green and compact in nature. The combination of 2,4-D with IAA was ineffective for the enhancement of the alkaloids in callus culture.

REFERENCES

Anitha, S. and B.D. Ranjitha Kumari, 2006a. Stimulation of reserpine biosynthesis in the callus of *Rauvolfia tetraphylla* L. by precursor feeding. Afr. J. Biotechnol., 5: 559-561.

Anitha, S. and B.D. Ranjitha Kumari, 2006b. Reserpine accumulation in NaCl treated calli of *Rauvolfia tetraphylla* L. Sci. Asia, 32: 417-419.

Anonymous, 1998. Indian Herbal Pharmacopoeia. In: A Joint Publication of Regional Research Laboratory, Jammu Tawi, Indian Drug Manufacturer's Association, Mumbai. Handa, S.S., D. Mundkinajeddu and A.K. Mangal (Eds.), Vol. 1, pp: 121-129.

Baskar Rajan, G., 2001. Biotechnological Advances in *Solanum eleagnifolium*-night Shade Plant. In: Role of Biotechnology in Medicinal and Aromatic Plants. Irfan A. Khan and Atiya Khanum (Eds.), Vol. 4, Ukkaz Publications, Hyderabad, India, pp: 126-132.

Charlwood, B.V., K.A. Charlwood and J. Molina-Torres, 1995. Accumulation of Secondary Compounds by Organised Plant Cultures. In: Secondary Products from Plant Tissue Culture. Charlwood, B.V. and M.J.C. Rhodes (Eds.), Clarendon Press, Oxford, pp: 167-200.

Dhiman, A.K., 2006. Ayurvedic Drug Plants. Daya Publishing House, New Delhi, pp: 343-345.

Faisal, M. and M. Anis, 2002. Rapid *in vitro* propagation of *Rauvolfia tetraphylla* L., an endangered medicinal plant. J. Physiol. Mol. Biol. Plants, 8: 295-299.

Holden, P.R., M.A. Holden and M.M. Yeoman, 1988. Variation in the Secondary Metabolism of Cultured Plant Cells. In: Manipulating Secondary Metabolism in Culture. Robins, R.J. and M.J.C. Rhodes (Eds.), Cambridge University Press, Cambridge, pp: 15-29.

Iwase, A., H. Aoyagi, M. Ohme-Takagi and H. Tanaka, 2005. Development of a novel system for producing ajmalicine and serpentine using direct culture of leaves in *Catharanthus roseus* intact plant. J. Biosci. Bioeng., 99: 208-215.

Lindsey, K., 1988. Prospects for the Genetic Manipulation of Complete Metabolic Pathways. In: Manipulating Secondary Metabolism in Culture. Robins, R.J. and M.J.C. Rhodes (Eds.), Cambridge University Press, Cambridge, pp: 123-136.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plants, 15: 473-497.

Narasimhan, S. and G.M. Nair, 2004. Release of berberine and its crystallization in liquid medium of cell suspension cultures of *Coscinium fenestratum* (Gaerth.). Curr. Sci., 86: 1369-1371.

Olivera, A.J.B., K.L.R.F.A. Machado and S.S.L. Kirszenzaft, 2001. Callus culture of *Aspidosperma ramiflorum* Muell. Arg.: Growth and alkaloid production. Marinza, 23: 609-612.

Pullaiah, T., 2002. Medicinal Plants in India. Vol. 2, Regency Publications, New Delhi, India, pp: 441-443.

Radwanski, E.R. and R.L. Last, 1995. Tryptophan biosynthesis and metabolism. Biochem. Mol. Gen. Plant Cell, 7: 921-934.

Ralphs, M.H. and D.R. Gardner, 2003. Distribution of norditerpene alkaloids in tall larkspur plant parts through the growing season. J. Chem. Ecol., New York, 29: 2013-2021.

Rhodes, M.J.C., J. Hamill, A.J. Parr, R.J. Robins and N.J. Walton, 1988. Strain Improvement by Screening and Selection Techniques. In: Manipulating Secondary Metabolism in Culture. Robins, R.J. and M.J.C. Rhodes (Eds.), Cambridge University Press, Cambridge, pp: 83-93.

Rocha, L.K., A.J.B. Olivera, C.A. Mangolin and M.F.P.S. Machado, 2005. Effect of different culture medium components on production of alkaloid in callus tissues of *Cereus peruvianus* (Cactaceae). Maringa, 27: 37-41.

Seth, R. and A.K. Mathur, 2005. Selection of 5-methyltryptophan resistant callus lines with improved metabolic flux towards terpenoid indole alkaloid synthesis in *Catharanthus roseus*. Curr. Sci., 89: 544-548.

Sheludko, Y.U., I. Gerasimenko, M. Unger, I. Kostenyuk and J. Stockigt, 1998. Induction of alkaloid diversity in hybrid plant cell lines. Plant Cell Rep., 18: 911-918.

Siah, C.L. and P.M. Doran, 1991. Enhanced codeine and morphine production in suspended *Papaver somniferum* cultures after removal of exogenous hormones. Plant Cell Rep., 10: 349-353.

Srividhya, N., B. Poorna Sridevi and P. Sathyanarayana, 1998. Azadirachtin and nimbin content in the *in vitro* shoots and roots of *Azadirachta indica* J. Juss. Indian J. Plant Physiol., 3: 129-134.

Swarup, R. and J.R. Arora, 2000. Plant tissue culture from research to commercialization: A decade of support. Department of Biotechnology, Government of India, New Delhi, pp: 48-49.

Yeh, F.T., W.W. Huang, C.C. Cheng, C. Na and H.S. Tsay, 1994. Tissue culture of *Dioscorea doryophora* Hance. II. Establishment of suspension culture and the measurement of diosgenin content. Chinese Agron. J., 4: 257-268.