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Influence of Growth Retardants on Serpentine Accumulation in *Catharanthus roseus* Cell Suspension Cultures

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Abstract: The effect of three growth retardants Chlormequat chloride, AMO 1618 and Tetcyclacis on accumulation of serpentine in *Catharanthus roseus* cell culture was studied. These products are known to inhibit gibberellin biosynthesis in the cell. Low and high concentrations of growth retardants were applied to *C. roseus* cultures and all of them affect growth and accumulation of serpentine. All of these substances lowered cell mass growth at high concentrations compared with control. All of tested compounds increased the content of serpentine compared to the control. High concentration of AMO-1618 resulted in two-fold increase in serpentine accumulation (25.94±1.5 μg g^{-1} FW) at day 8 after treatment in the cells and the secretion of serpentine to the medium was decreased with time. Chlormequat chloride at high concentration resulted in serpentine productivity of 18.6±0.8 μg g^{-1} FW at day 1, but no effect on secretion in the medium. Tetcyclacis resulted in lysis of the cells and consequently release of serpentine to the medium.

Key words: *Catharanthus roseus*, cell culture, fresh weight, growth retardants, indole alkaloids, ajmalicine, serpentine

INTRODUCTION

Gibberellins are diterpenoid acids that act as plant growth regulators. Gibberellins are essential for seed germination, stem elongation promotion and in some plants it controls flowering (Hardtke, 2003; Sponsel, 1988). Previous studies have shown that gibberellin has an inhibitory effect on secondary metabolites production. Addition of gibberellin to hairy roots of *Solanum aviculare* enhanced the growth but reduced the specific steroidal-alkaloid levels (Subroto and Doran, 1994). Also gibberellin strongly inhibited hyoscyamine accumulation in transformed root cultures of *Hyoscyamus muticus* without changes in root morphology (Vanhala *et al*., 1998). In *Catharanthus roseus* cell cultures treatment with gibberellic acid strongly inhibited ajmalicine accumulation (Carpin *et al*., 1997).

Plant growth retardants are synthetic compounds, antagonistic to gibberellins and auxins. Four different types of these inhibitors are known, among them onium compounds such as Chlormequat Chloride (CCC) and Amo-1618 and N-containing heterocyclic compounds such as Tetcyclacis (TC). There is general agreement that application of these compounds to seedlings inhibits stem growth (Nes *et al*., 1982; Rademacher, 2000). Chlormequat Chloride (CCC) and AMO-1618 inhibit copalyl diphosphate synthase and tetcyclacis inhibits the cytochrome P450 monooxygenase enzyme catalyzing the oxidative step from ent-kaurene to ent-kaurenoid acid in the pathway of gibberellin biosynthesis.

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Catharanthus roseus, a tropical plant known as Madagascar periwinkle produces several important medicinal compounds. *C. roseus* has been widely studied for its production of the anticancer drugs vinblastine and vincristine, as well as the antihypertensive compounds ajmalicine and serpentine (Moreno *et al*., 1995). For many years, plant cell and tissue cultures have been investigated as an alternative source of production but low yield and absence of some precursors are the key bottlenecks to this technology. The use of abiotic and biotic elicitors is a promising tool to improve the yields of products in cell culture systems (Moreno *et al*., 1995; Verpoorte *et al*., 1997; El-Sayed and Verpoorte, 2007). Regulation of production of *C. roseus* alkaloids for commercial use can be induced developmentally or by exogenous signals. Direct cultures as a new tool to overcome metabolite production instability of *C. roseus* alkaloids promoted the productivity of ajmalicine and serpentine (Iwase *et al*., 2005). Application of various exogenous chemicals can improve the alkaloid production of *C. roseus*. Treating *C. roseus* plants with either triadimefon or ketokenozale compounds which are similar to the used growth retardants of this study resulted in an increase of the content of ajmalicine, the precursor of serpentine (Abdul Jaleel *et al*., 2006; Abdul Jaleel *et al*., 2007).

Here we report on the effect of three growth retardants (CCC, Amo-1618 and TC) on the growth of a *C. roseus* cell suspension culture as well as on the accumulation of the indole alkaloid serpentine in the cultures.

MATERIALS AND METHODS

Cell Line and Culture Conditions

Catharanthus roseus cell suspension culture line (CREG) accumulating serpentine was established from a leaf explant of the Egyptian cultivar (No.20000003) on B5 medium in June 2000. The medium contains B5 salts (Gamborg *et al*., 1968), 100 mg L^{-1} myo-inositol, 10 mg L^{-1} thiamine, 1 mg L^{-1} pyridoxine, 1 mg L^{-1} nicotinic acid, 1.86 mg L^{-1} NAA and 20 g L^{-1} sucrose. The friable callus was then transferred to liquid medium under the same conditions to initiate the cell suspension and sub-cultured every two weeks. Cultures were placed on a gyratory shaker at 110±5 rpm and at 25±1°C, with 24 hours light (14-25 μmol m^{-2} sec^{-1}). For the experiment, 1.5 g of filtered cells were transferred to a 150 mL Erlenmeyer flask containing 20 mL of medium under sterile conditions. The experiment was performed in duplicate.

Preparation of Growth Retardants

The growth retardants (CCC, Amo-1618 and tetcyclacis) were obtained from the Department of plant physiology, Wageningen University, The Netherlands. They were prepared by dissolving them in the smallest possible amount of 70% ethyl alcohol (not more than 1 mL), then completed with water to the required volume and added to the cultures after filter sterilization. CCC was applied in final concentrations of 1 mM, 5 mM and 10 mM. Amo-1618 was applied in concentrations of 5, 10 and 50 μM while TC was applied in concentrations of 0.1, 0.5 and 1 mM. Control cultures received the same amount of alcohol.

Harvesting of the Cultures

The cultures were harvested on days 1, 3 and 8 after treatment with growth retardants. The biomass was separated from medium using a glass filter, weighed, frozen in liquid nitrogen and stored in -80°C.

Sample Extraction and HPLC Analysis of Serpentine

Frozen biomass (125 mg in 1.5 mL Eppendorf vial) was extracted using a pestle and sea sand with 350 μL of 0.1% trifluoroacetic acid solution. The vial was then sonicated for 30 min and centrifuged at 13,000 rpm for 15 min. The supernatant was used for alkaloid determination.

One hundred microliter of cell extract supernatant or centrifuged medium were analyzed by HPLC equipped with a photo-diode array detector. A C18 RP Vydac column (no. 218 MS54, 4.6*250 mm, USA) was used with an isocratic elution system of trifluoroacetic acid: acetonitrile: water (0.1:21:79) at a flow rate of 1 mL min^{-1}.

Peak identification was based on a comparison of retention time and UV spectra with authentic standard of serpentine.

RESULTS AND DISCUSSION

Different concentrations of three growth retardants (CCC, Amo-1618 and tetcyclacis) were applied to the cell cultures of *C. roseus*. Low concentrations of these substances did not affect much the growth of the cells. However higher concentrations of the growth retardant tetcyclacis resulted in low growth and at day 8 most of the cells were dead (Table 1). Application of high concentrations of tetcyclacis was reported to reduce cell division in cell suspension cultures of maize, leading to qualitative and quantitative changes in the produced sterols (Rademacher, 2000).

Effect of CCC on Serpentine Contents

Cell cultures treated with CCC did not show differences in the amount of serpentine in the media compared to controls (Table 2). Serpentine accumulation in the cells slightly increased with the time starting at 13.5±0.6 to 17.02±0.4 μg g^{-1} FW at a concentration of 1 mM. Application of higher

Table 1: Effect of growth retardants on fresh weight (g) of *Catharanthus roseus* cell suspension

	Days		
Treatments	1	3	8
Control	2.30±0.055	3.45±0.15	4.35±0.45
CCC			
1 mM	2.18±0.00	2.63±0.02	3.00±0.005
5 mM	2.02±0.10	2.50±0.03	2.90±0.15
10 mM	2.00±0.005	2.40±0.035	2.33±0.20
AMO-1618			
5 μM	2.47±0.07	3.17±0.03	4.05±0.15
10 μM	2.58±0.08	3.33±0.03	4.00±0.10
50 μM	2.35±0.15	2.92±0.10	3.20±0.30
TC			
0.1 mM	2.34±0.005	2.46±0.10	4.29±0.13
0.5 mM	2.17±0.020	2.85±0.05	4.15±0.08
1.0 mM	2.20±0.015	2.80±0.20	0.80±0.05

Table 2: Effect of growth retardants on serpentine accumulation (μg g^{-1} FW) in cells and media of *C. roseus* suspension cultures

	Medium			Cells		
	1	3	8	1	3	8
Treatments	days			days		
Control	0.30±0.050	0.36±0.050	0.30±0.050	11.90±0.40	9.90±0.30	10.50±0.30
CCC						
1 mM	0.30±0.048	0.30±0.046	0.31±0.047	13.50±0.60	15.60±0.60	17.02±0.40
5 mM	0.35±0.050	0.36±0.050	0.30±0.047	15.06±0.04	12.80±0.10	11.90±1.01
10 mM	0.35±0.047	0.38±0.048	0.35±0.046	18.60±0.80	14.70±0.30	13.50±1.00
AMO-1618						
5 μM	0.27±0.010	0.17±0.020	0.12±0.003	13.30±0.54	11.01±0.70	16.80±0.07
10 μM	0.21±0.002	0.14±0.010	0.12±0.010	11.95±0.30	11.65±0.13	21.85±2.00
50 μM	0.20±0.013	0.18±0.010	0.10±0.007	13.40±1.70	12.60±0.01	25.94±1.50
TC						
0.2 mM	0.44±0.041	0.41±0.048	0.37±0.047	15.94±0.96	12.52±2.13	7.53±2.44
0.5 mM	0.40±0.049	0.46±0.052	0.36±0.047	18.05±1.50	13.50±1.23	8.35±0.60
1.0 mM	0.41±0.049	0.40±0.049	0.82±0.190	14.50±1.80	12.09±0.55	10.74±1.17

concentrations of CCC resulted in fast increase of serpentine at day 1 especially at concentration of 10 mM where 18.6±0.8 μg g^{-1} FW was found, but at day 3 a decrease was observed. At day 8 the level was still higher than in the controls. It was reported that CCC stimulates taxol production in *Taxus brevifolia* trees bark in an 8-week treatment period (Strobel *et al*., 1994).

Effect of Amo-1618 on Serpentine Contents

Amo-1618 showed the same effect on serpentine content in the media in all concentrations (Table 2). The amount of serpentine decreased gradually one day after treatment till day 8. In contrast to the medium pattern, serpentine amount in the cells is increasing with time. A two-fold increase was observed at day 8 of the 50 μM treatment (25.94±1.5 μg g^{-1} FW). This experiment was repeated and followed for a longer time to see if a continuous increase could be achieved but serpentine content declined after day 8 (data not shown). AMO-1618 applied to tobacco seedlings caused an accumulation of 2,3-oxidosqualene and inhibited the incorporation of radiolabeled mevalonic acid into sterols (Douglas and Paleg, 1978; Nes *et al*., 1982).

Effect of Tetcyclacis (TC) on Serpentine Contents

Application of TC at different concentrations to the cells resulted in a slight increase in serpentine content at the first day followed by a gradual decrease (Table 2). At day 8, 1mM concentration treatment led to the death of the cells and the amount of serpentine increased in the medium to a maximum value (0.82±0.19 μg mL^{-1}) as a result of cells lysis. The 0.5 mM concentration of TC gave a production of serpentine of 18.05±1.5 μg g^{-1} FW at day 1 compared to 11.9±0.4 μg g^{-1} FW in control at the same day. As TC is a blocker of cytochrome P450-dependent monooxygenases, biosynthesis of flavonoids and other phenylpropanoids was inhibited by relatively high dosages of TC in a cell-free system of soybean cell cultures (Forkmann and Heller, 1999; Weisshaar and Jenkins, 1998). In *Catharanthus roseus*, alkaloid accumulation in cell suspension culture was decreased after ketoconazole treatment which acts similar as TC (Contin *et al*., 1999).

CONCLUSION

Inhibitors of GA biosynthesis can be used as inducers of indole alkaloids productivity in cell cultures of *C. roseus*. Low concentrations of growth retardants did not affect biomass accumulation. AMO-1618 is the best treatment among others to induce two-fold serpentine accumulation in the cells per gram FW. Tetcyclacis has a clear effect on cell growth and serpentine content.

REFERENCES

Abdul Jaleel, C., R. Gopi, G.M.A. Lakshmanan and R. Panneerselvam, 2006. Triadimefon induced changes in the antioxidant metabolism and ajmalicine production in *Catharanthus roseus* (L.) G. Don. Plant Sci., 171: 271-276.

Abdul Jaleel, C., P. Manivannan, B. Sankar, A. Kishorekumar, R. Gopi, R. Somasundaram and R. Panneerselvam, 2007. Induction of drought stress tolerance by ketoconazole in *Catharanthus roseus* is mediated by enhanced antioxidant potentials and secondary metabolite accumulation. Colloids and Surfaces B: Biointerfaces (In Press).

Carpin, S., L. Ouelhazi, M. Filali, J.C. Chenieux, M. Rideau and S. Hamdi, 1997. The relationship between the accumulation of a 28 kD polypeptide and that of indole alkaloids in *Catharanthus roseus* cell suspension cultures. J. Plant Physiol., 150: 452-457.

Contin, A., G. Collu, R. Van der Heijden and R. Verpoorte, 1999. The effects of phenobarbital and ketoconazole on the alkaloid biosynthesis in *Catharanthus roseus* cell suspension cultures. Plant Physiol. Biochem., 37: 139-144.

Douglas, T.J. and L.G. Paleg, 1978. Amo 1618 and sterol biosynthesis in tissues and subcellular fractions of tobacco seedlings. Phytochemistry, 17: 705-712.

El-Sayed, M. and R. Verpoorte, 2007. *Catharanthus* terpenoid indole alkaloids: Biosynthesis and regulation. Phytochem. Rev., 6: 277-305.

Forkmann, G. and W. Heller, 1999. Biosynthesis of Flavonoids. In: Comprehensive Natural Products Chemistry. Barton, D., K. Nakanishi and O. Meth-Cohn (Eds.), Elsevier, Amsterdam, pp: 713-748.

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 155-158.

Hardtke, C.S., 2003. Gibberellin signaling: GRASs growing dispatch roots. Curr. Biol., 13: R366-R367.

Iwase, A., H. Aoyagi, M. Ohme-Takagi and M. 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.

Moreno, P.R.H., R. Van der Heijden and R. Verpoorte, 1995. Cell and tissue cultures of *Catharanthus roseus*: A literature survey. II Updating from 1988 to 1993. Plant Cell Tiss. Org. Cult., 42: 1-25.

Nes, W.D., T.J. Douglas, J.T. Lin, E. Heltmann and L.G. Paleg, 1982. Regulation of isopentenoid biosynthesis by plant growth retardants in *Nicotiana tabacum*. Phytochemistry, 21: 575-579.

Rademacher, W., 2000. Growth retardants: Effect on gibberellin biosynthesis and other metabolic pathways. Ann. Rev. Plant Physiol. Plant Mol. Biol., 51: 501-531.

Sponsel, V.M., 1988. Gibberellin Biosynthesis and Metabolism. In: Plant Hormones and Their Role in Plant Growth and Development. Davies, P.J. (Ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands.

Strobel, G., A. Stierle and W.M. Hess, 1994. The stimulation of taxol production in *Taxus brevifolia* by various growth retardants. Plant Sci., 101: 115-124.

Subroto, N.A. and P.M. Doran, 1994. Production of steroidal alkaloids by hairy roots of *Solanum aveculare* and the effect of gibberellic acid. Plant Cell Tiss. Org. Cult., 38: 93-102.

Vanhala, L., M. Eeva, S. Lapinjoki, R. Hiltunen and K.M.O. Caldentey, 1998. Effect of growth regulators on transformed root cultures of *Hyoscyamus muticus*. J. Plant Physiol., 153: 475-481.

Verpoorte, R., R. Van der Heijden and P.R.H. Moreno, 1997. Biosynthesis of Terpenoid Indole Alkaloids in *Catharanthus roseus* Cells. In: The Alkaloids. Cordell, G.A. (Ed.), Academic Press, San Diego, 49: 221-299.

Weisshaar, B. and G.I. Jenkins, 1998. Phenylpropanoid biosynthesis and its regulation. Curr. Opin. Plant Biol., 1: 251-257.