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EFFECT OF CULTIVARS AND YEAST EXTRACT ON KEY PRIMARY AND SECONDARY METABOLITES IN *Catharanthusroseus*(L) G. DON.

Mohamed, Z.A.; M.M.M. Darwish and Najme A. Said

AgriculturalBotany Department, Faculty of Agriculture, Mansoura University, Mansoura, Egypt

ABSTRACT

A pot experiment was conducted during the growing seasons 2013/2014 and 2014/2015 at The Experimental Farm and laboratories of the Agricultural Botany Department, Faculty of Agriculture, Mansoura University to study the effects of yeast extract either at 0, 4 or 8 gL^{-1} on key primary and secondary metabolites of *Catharanthusroseus*, cultivars cv. *rosea* and cv. alba. Results indicated that total alkaloids, total soluble phenols and flavonoids were significantly higher in cv*rosea* compared with cv*alba*. Applicationof yeast extract (YE), generally, enhanced the accumulation of alkaloids, phenols and flavonoids. In addition, treatment with YE increased the concentration of kinetin, indole acetic acid, gibberellic acid and benzyladenine whereas decreased that of abscisic acid. Moreover, the concentration of the essential elements N, P, K, Ca and Mg were higher in YE-treated plants, and, the effect of the higher level was more effetine. It was concluded that YE could be utilized as an elicitor to enhance the accumulation of the medicinally-important secondary metabolites in *C. roseus* plants.

INTRODUCTION

Catharanthusroseus(L) G. Don is a tropical and subtropical species belonging to Apocyanaceae. *C. roseus* is a renowned medicinal plant due to the presence of alkaloids which is distributed in all parts of the plant. The plant species contains about 130 alkaloids of indole group and many other secondary metabolites including monoterpenoids, glucosides, steroids, phenolics, flavonoids and anthocyanins. The plant is used for the treatment of diabetes, fever, malaria, throat infections, and chest complaints, for the regulation of menstrual cycles and as euphoriant (Ambusta, 1992). The medicinal value of the plant is mostly due to its alkaloids, though this content is considerably low. So, many strategies have been attempted to enhance its alkaloids content. Elicitation have been a widely adopted approach of enhancing secondary metabolite production in general and specifically for inducing the biosynthesis of *C. roseus* alkaloids. Elicitors are either biotic or abiotic. Abiotic elicitors include heavy metal ions, inhibition of some metabolic steps, certain antibiotics, UV radiation, stress factors and growth substances. Treatment with lead was reported to increase content of alkaloids, flavonoids and phenols in *C. roseus* callus cultures (Amirjani*et al*., 2015). Tryptophan and Putescine applied as a foliar spray increased total alkaloids content in *C. roseus* leaves (Talaat*et al*., 2005). In addition, salinity stress (Jaleel *et al*., 2008, b) and various growth substances (Jaleel *et al*., 2008, a; Alam*et al*., 2012) have been used to augment alkaloids biosynthesis. Biotic elicitors are complex biological compounds with unknown composition like microbial cell wall preparations and yeast extract (YE). YE is used as a biotic elicitor for the induction and enhancement of secondary metabolites (Abraham *et al*., 2011). YE was reported to enhance silymarin production in cell cultures of *Silybummarianum* (Sanchez-Sampedro*et al*., 2005; Hasanloo*et al*., 2008), the alkaloid Mitragynine content in *Mitragynaspeciosa* suspension cultures (Zuldin*et al*., 2013), Isoflavone content in soybean (Al-Tawaha, 2011), and noradrenaline production in hairy root culture of *Portulacaeoleracea*(Pirian and Piri, 2013). However, the effect of YE on elicitation of secondary metabolites in *C. roseusin vivo* is less investigated and poorly understood. So, the aim of the present study was to investigate the effect of YE on key metabolites in *C. roseusin vivo*.

MATERIALS AND METHODS

Two pot experiments were conducted during the two successive growing seasons 2013/2014, and 2014/2015at The Experimental Farm and Laboratories of the Agric. Bot. Dept., Fac. of Agric., Mansoura University, to study the effects of yeast extract (YE) either at 0, 4 or 8 g L^{-1}on certain secondary metabolites, growth substances and some macro-elements of two cultivars of *Catharanthusroseus*nemely cv *rosea* and cv*alba*. *Catharanthusroseus* seeds were collected from the Campus gardens of Mansoura University and were surface sterilized in a 0.2% $HgCl_2$ solution for 5 min, then thoroughly washed with tap water, and sown on10th of March and 28 th March in the two growing seasons respectively, in plastic pots, 25 cm in diameter containing 7 kg of a soil, 20 seeds/ pot.Main physical and chemical characteristics of the experimental soil shown in Table (1) were estimated according to Hoddinott and Lamb (1990).

Table (1). Physical and chemical characteristics of the experimental soil.

Sand %	Silt %	Clay	Organic matter %	Total N %	Available K ppm	Available P ppm	TSS
38.6	26.2	35.2	2.1	0.13	226	13	0.24

Pots were irrigated to maintain field capacity and arranged in a complete randomized block design with four replications. Thirty days after sowing, seedlings were thinned to leave four uniform seedlings per pot. 45

days after sowing (DAS), yeast extract was sprayed onto foliage till leavesdripping using tween 20, 0.05%, as a wetting agent. Control plants were sprayed with deionized water. One week after the first YE application, a second application was done using the same concentrations. Each pot received as calcium superphosphate (15.5 % P_2O_5) at the rate of 3 g/ Pot mixed with the soil before sowing. 2 g ammonium sulphate(20.5 % N) and 2 g of potassium sulfate (50 % K_2O)were also added to each pot in two equal doses, 25, 40 DAS. 65 DAS, shoot samples were collected to determine the following biochemical constituents.

Total alkaloids:

Concentration of total alkaloids was determined according to the method of Afaq*et al*. (1994). Five hundred mg of shoots powder were transferred to a 100 ml round bottom reflux flask and refluxed for 6 h in a known volume of ethyl alcohol. The extract was then filtered using whatman filter paper No. 1 and 50 ml of dilute HCl was added. Afterward, it was transferred to a separating funnel to which 50 ml of diethyl ether was added.The mixture was shaken for 15 min, the upper diethyl ether layer was discarded whereas the lower water layer was decanted in a beaker and made slightly basic using ammonia solution. The decanted content was again fractionated in a separating funnel using 50 ml of diethyl ether. To the second decant, anhydrous sodium carbonate was added. The mixture was decanted in a pre-weighed dry porcelain dish and was heat-evaporated until dry and weighed again. Total alkaloids concentration was expressed as mg g^{-1} dry matter.

Total flavonoids:

Total flavonoids concentration was determined by aluminium chloride colorimetric method as described by Lin and Tang (2007). One gram of the methanolic extract was mixed with 0.1 ml of 10% aluminium chloride hexahydrate, 0.1 ml of 1 M potassium acetate and 2.8 ml of deionized water. After incubation for 40 min at room temperature, absorbance of the reaction mixture was recorded at 415 nm. Total flavonoids concentration was estimated from a standard curve established using quercetin and expressed as mg (quercetin equivalent) per g dry matter.

Total Soluble phenols:

Total Soluble phenols concentration was determined using Folin-Ciocalteu reagent according to Singleton *et al*. (1999). The reaction mixture contained 1 ml of the methanolic extract, 9 ml of distilled water, 1 ml of Folin-Ciocalteu reagent and 10 ml of 7% (w/v) sodium carbonate, and incubated for 90 min at room temperature. Absorbance was recorded at 765 nm, and total phenols concentration was estimated using a standard curve established with Gallic acid and expressed as mg (Gallic acid equivalent) per gram dry matter.

Endogenous plant hormones:

Extraction of Endogenous plant hormones were carried out according to the method of Shindy and Smith (1975) and determined using HPLC procedures as described by Baydar and Ulger (1998). For extraction, 6 g of the fresh shoot samples were homogenized and extracted in cold methanol (80% v/v). The extract was evaporated to the aqueous phase in a rotary evaporator. The aqueous phase was adjusted to pH 8.6 with 1% NaOH and partitioned three times with equal volumes of ethyl acetate. After removalof the ethyl acetate phase, the aqueous phase was adjusted to pH 2.8 with 1% HCl and partitioned three times with equal volumes of ethyl acetate. The combined acidic ethyl acetate phase was used for HPLC determination of acidic endogenous plant hormones. The dried basic ethyl acetate fraction was dissolved in 80% methanol which is then evaporated under vacuum, leaving an aqueous phase which was adjusted to pH 2.8 with 1% HCl and partitioned three times with 50 ml of ethylacetate.The ethyl acetate phases were combined, reduced to 5 ml volume and used for the determination of neutral auxins. The remaining aqueous phase was adjusted to pH 5.5 with 1% NaOH and partitioned three times with 50 ml of water-saturated L-butanol. Butanol phases were combined, reduced to 5ml volume and used for the determination of cytokinins.

For HPLC determination of plant growth substances, 20 µl of sample was injected into HPLC (Waters U6K HPLC). Separation and determination were performed on a C_{18} column (3.9 x 300 mm, silica-based packing material). The elution system consisted of 100% methanol, 2% acetic acid and was run at a flow rate of 1.0 ml min^{-1}.

Determination of elements concentration:

200 mg of the dried shoot powder was wet digested in a mixture of sulfuric and perchloric acids (2:1 v/v). Total nitrogen was determined using modified Microkjeldahl's method according to Pregl (1945). Phosphorus was determined by the molybdenum blue method according to Murphy and Riley (1962).Potassium concentration was determined by the flamephotometric method, whereas Ca and Mg concentration were determined using versenate method in the wet digested plant material according to Richards (1954).

Statistical analysis:

ANOVA was performed using SPSS (version 16.0) as a combined analysis of the two growing seasons. Duncan's Multiple Range list was applied to determine significant difference between means when ANOVA was significant at $P \leq 0.05$.

RESULTS

Alkaloids, Total soluble phenols and flavonoids concentrations were significantly higher in cvrosea compared with cv alba. RoseaCV contained 46.5, 54.2, 34.5% higher alkaloids, total soluble phenols and flavonoids, respectively compared with cv alba. YE increased alkaloids, total soluble phenols and flavonoids concentration, though the increase was not significant in the case of alkaloids and total soluble phenols (Table 2). In its enhancing effect on the concentration of flavonoids, there was no significant difference between the two tested levelsof YE. The interaction between cultivars and YE was not significant regarding the

concentration of alkaloids, Total soluble phenols and flavonoids.

The concentration from endogenous plant hormones differed significantly between the two cultivars. Roseacv contained higher concentration from IAA, GA_3, kinetin (kin) and Benzyl Adenine (BA). On the other hand, cvrosea had lower concentration from abscisic acid (ABA). YE at both applied levels increased Kin whereas decreased abscisic acid concentration (Table 3). On the other hand, IAA, GA_3 and BA concentration were increased only at the higher adopted level. Though concentration of IAA, GA_3, Kin, BA were higher at the higher YE level ($8gL^{-1}$) compared with those at the lower level ($4\ gL^{-1}$), there was no significant difference between the two levels. This is also true in the case of ABA, though its concentration was lower at the higher level. The interaction between cultivars and YE was significant in case of Kin and BA concentration. The highest Kin concentration was recorded in cvrosea treated with YE at the higher level, whereas the lowest concentration was recorded in cv albauntreated with YE. Similar trend was recorded regarding BA concentration, where cvrosea treated with YE at its higher level contained the highest BA concentration whereas cv alba not treated with YE contained the lowest level.

Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) concentration were higher in cv rosea compared with cv alba, though the effect was insignificant in case of nitrogen concentration.YE increased N, P, K, Ca and Mg concentration though the effect was insignificant at the lower level (Table 4). YE at the higher level led to a significant higher concentration from both N, P compared with the lower level. The difference between the two levels regarding K, Ca and Mg was insignificant. The effect of the interaction between cultivars and YE was insignificant regarding the concentration of all elements except Mg. Cultivar rosea treated with the higher level of YE contained the highest Mg concentration whereas the lowest Mg concentration was recorded in cv alba not treated with YE.

Table (2). Effects of yeast extract on total alkaloids, phenols and flavonoids in *C. roseus* shoots.

Character	Treatment	Alkaloids mg g^{-1} D. wt.	Phenols mg g^{-1} D. wt.	Flavonoids mg g^{-1} D. wt
A	A_1	2.73	14.82	81.10
	A_2	4.00	22.85	109.08
Significance at 0.05		*	*	*
B	B_o	3.03	18.25	80.66^{b}
	B_1	3.31	18.35	97.209^{b}
	B_2	3.76	19.90	107.41^{a}
Significance at 0.05		N.S.	N.S.	*
A x B	A_1B_o	2.48	14.75	70.75
	A_1B_1	2.78	14.55	82.25
	A_1B_2	2.95	15.15	90.30
	A_2B_o	3.58	21.75	90.85
	A_2B_1	3.85	22.15	112.15
	A_2B_2	4.58	24.65	124.53
Significance at 0.05		N.S	N.S.	N.S.

*** Means followed by the same letter are not significantly different at P = 0.05; NS = not significant**

Table (3). Effects of yeast extract on plant growth substances in *C. roseus*shoots.

Treatment	Character	IAA µg 100 g^{-1}F.wt	GA_3 µg 100 g^{-1}F.wt	Kinetin	BA	ABS µg 100 g^{-1}F.wt
A	A_1	13.92	18.68	11.15	0.41	7.63
	A_2	21.57	27.09	16.46	0.70	4.53
Significance at 0.05		*	*	*	*	*
B	B_o	14.39^{b}	20.25^{b}	9.25^{b}	0.42^{b}	7.76^{a}
	B_1	17.45^{ab}	22.96^{ab}	14.58^{a}	0.56^{ab}	5.55^{b}
	B_2	21.39^{a}	23.44^{a}	17.59^{a}	0.69^{a}	4.91^{b}
Significance at 0.05		*	*	*	*	*
A x B	A_1B_o	10.15	16.15	8.75^{c}	0.35^{c}	9.78
	A_1B_1	13.65	18.85	11.23^{c}	0.42^{c}	6.95
	A_1B_2	17.95	21.03	13.48^{bc}	0.48^{c}	6.15
	A_2B_o	18.63	24.35	9.75^{c}	0.49^{c}	5.75
	A_2B_1	21.25	27.08	17.93^{ab}	0.71^{b}	4.15
	A_2B_2	24.83	29.85	21.70^{a}	0.90^{a}	3.68
Significance at 0.05		N.S.	N.S.	*	*	N.S.

*** Means followed by the same letter are not significantly different at P = 0.05; NS = not significant**

Table (4). Effects of yeast extract on macroelements in *C. roseus*shoots.

Treatment	Character	Nitrogen mg g^{-1} D. wt.	Phosphorus mg g^{-1} D. wt	Potassium mg g^{-1} D. wt.	Calcium mg g^{-1} D. wt.	Magnesium mg g^{-1} D. wt.
A	A_1	30.80	3.54	30.39	2.74	0.51
	A_2	33.68	5.04	35.96	3.25	0.70
Significance at 0.05		N.S.	*	*	*	*
B	B_o	26.24^b	3.21^b	29.51^b	2.30^b	0.47^b
	B_1	29.20^b	4.23^b	33.46^{ab}	3.03^{ab}	0.55^{ab}
	B_2	41.38^a	5.44^a	36.55^a	3.68^a	0.78^a
Significance at 0.05		*	*	*	*	*
A x B	A_1B_o	24.80	2.78	26.33	2.08	0.42^b
	A_2B_1	28.33	3.33	30.63	2.88	0.51^b
	A_1B_2	39.28	4.53	34.33	3.28	0.59^b
	A_2B_o	27.48	3.65	32.80	2.53	0.53^b
	A_2B_1	30.08	5.13	36.30	3.18	0.60^b
	A_2B_2	43.48	6.35	38.78	4.08	0.98^a
Significance at 0.05		N.S.	N.S.	N.S.	N.S.	*

*** Means followed by the same letter are not significantly different at P = 0.05; NS = not significan**

DISCUSSION

The higher alkaloids concentrationin cv *rosea* compared with cv alba may be attributed to higher activities of carbonic anhydrase and nitrate reductase as well as higher leaf nitrogen concentration in cv *rosea* (Idrees*et al*., 2010). Dutta *et al*. (2005) pointed out that cultivar variations in *C. roseus*on the basis of alkaloids concentration is regulated at the level of gene expression.

The use of elicitors is an effective approach for inducing the production of alkaloids in plant tissues (Gautom*et al*., 2011). Elicitors may be biotic or abiotic. Various biotic elicitors have been employed to enhance alkaloids biosynthesis in *C. roseus*. Within this class of elicitors cell wall filtrates of the fungus *Protomycesgravidus* (Bhagwath and Hjortso(2000), *Pseudomonas fluorescens* (Jaleel *et al*., 2009), a combination of *P. fluorescens* and *Azospirillumbrasilense* (Karthikeyan*et al*., 2009), arbuscularmycorrhizal fungi, AMF (Karthikeyan*et al*., 2009) and cell wall fragments of *Aspergillusniger*, *Fusariummoniliforme* and *Trichodermaviride* (Namedo*et al*., 2002).

Low yield of *C. roseus* alkaloids versus their high demand worldwide led researchers to try diverse approaches to increase their production. Among these approaches utilization of endophytes (Koul*et al*., 2013), exogenous application of tryptophan and putrescine (Talaat*et al*., 2005), inductionof stress through salinization (Jaleel *et al*., 2008b), application of plant growth regulators and fungicides (Jaleel *et al*., 2008a), application of diverse growth regulators (Alam*et al*., 2012), and induction of stress through application of heavy metals (Amirjani*et al*., 2015).

Application of YE enhanced secondary metabolites production in *C. roseus*(Table 2). Similar results were reported in different plant species (Sanchez-Sampedro*et al*., 2005; Hasanloo*et al*., 2008; Al-Tawaha, 2011, Zuldin*et al*., 2013; Pirian and Piri, 2013). In *Curcuma mangga* cultures, YE induced the accumulation of secondary metabolites (Abrahim*et al*., 2011). YE enhanced the accumulation of the alkaloid 6-methoxymellein in carrot cells (Guo and Ohta, 1994). In addition, YE increased alkaloids yield in *Hyoscyamusmuticus* callus (Ibrahim *et al*., 2009). In *Mitragynaspeciosa*, YE inoculation to cell suspension cultures increased the production of the alkaloid mitragynine and the highest content was achieved at 250 mgl^{-1} YE (Zuldin*et al*., 2013). Likewise, YE increased hoscyamine 2.5-fold when added to *Daturastramonium* cultures (Zabetakis*et al*., 1999).

Simone (2010) reported that the elicitation of terpeneindole alkaloids (TIA) through the application of YE was accompanied with the induction of reactive oxygen species (ROS). Also, the author ascribed YE-induced TIA to enhanced expression of TIA-biosynthetic genes. The generation of ROS through oxidative burst was also reported in tobacco cultures elicited with YE (Baier*et al*., 1999). Atransient increase in cytosolic calcium levels in *C. roseus* cells was reported by Memelink*et al*. (2001), in harmony with the results of the present investigation (Table 4). Induction of Ca^{+2} is necessary for the induction of Jasmonate accumulation as well as for strictosidine synthase (STR) and tryptophan decarboxylase (TDC) gene expression (Memelink*et al*., 2001), both effects lead to enhanced TIA biosynthesis. Methyl Jasmonate (MJ) is a general secondary metabolism inducer and enhanced tabersonine biosynthesis in hairy root cultures of *C. roseus* (Rodriguez *et al*., 2003). In addition, the perception of YE in *C. roseus*suspension cultures leads to the induction of TIA biosynthesis genes including those encoding for STR and TDC (Pauw*et al*., 2004).

It is worth mentioning that the involvement of the elicitor in the course of essential events in secondary metabolism proceeds as follows (Siddiqui *et al*., 2013 as follows Siddiqui et al 2013):

1) The elicitor binds to plasma membrane receptors.
2) Influx of Ca^{2+} to the cytoplasm.
3) Cytoplasm pH decreases whereas protein phosphorylation patterns increases and the activity of NADPH oxidases and protein kinase is increased.
4) Cell wall structure changes towards enhanced lignification and ROS generation enhances.

5) Synthesis of JA and salicyclic acid as secondary messengers is enhanced.
6) Genes that produce defence-related proteins, plant defense molecules like phytoalexins and other secondary compounds including alkaloids are enhanced.

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تأثير اصناف ومستخلص الخميرة على بعض مركبات التمثيل الحيوى الأولية و الثانوية الهامة فى نبات الونكا

زين العابدين عبد الحميد محمد، محمود محمد مصطفى درويش و نجمة عبد السلام سعيد السائح
قسم النبات الزراعى، كلية الزراعة، جامعة المنصورة، المنصورة، ج.م.ع

اجريت التجربة خلال الموسمين ٢٠١٣/٢٠١٤ و ٢٠١٤/٢٠١٥ في المزرعة التجريبية ومعامل قسم النبات الزراعي، كلية الزراعة جامعة المنصورة لدراسة تاثير مستخلص الخميرة بتركيز ٤ و ٨ جم/لتر على بعض مركبات التمثيل الحيوى الأولية و الثانوية الهامة فى نبات الونكا ، صنفىRosea وصنف Alba. وأشارت النتائج إلى ان القلويدات الكلية، الفينولات الذائبة الكلية والفلافونيدات كانت أعلى تركيزاً في صنف Roseaمقارنة مع صنف Alba. وأشارت النتائج أيضاً إلى انالمعاملة بمستخلص الخميرة،بصفة عامة،تؤدى إلى زيادة تراكم القلويدات الكلية، الفينولات الذائبة الكلية والفلافونيداتوكذا زيادة تركيز الكينتين، إندولحمض الخليك ، حمض الجبريليك و البنزيلالأدينين بينما تؤدى إلى نقص فى تركيز حمض الأبسيسيك. وعلاوة على ذلك، فإن تركيز العناصر الأساسية النيتروجين، الفوسفور، كانت اعلى في النباتات المعاملة بمستخلص الخميرة. وكان تأثير المستوى الاعلى من المستخلص (٨جم/لتر) هوالأكثر فعالية فى هذا الشأن.

وفى ضوء النتائج المتحصل عليها يمكن إستنتاج أن مستخلص الخميرة يمكن ان يستخدم لتحسين تراكم المركبات الثانوية ذات الأهمية الطبية في نبات الونكا.