AcademicPres

Available online at www.notulaebiologicae.ro

Print ISSN 2067-3205; Electronic 2067-3264

Not Sci Biol, 2013, 5(4):458-461

Notulae Scientia Biologicae

Analysis of Several Popular Cultivars of Madagascar Periwinkle (*Catharanthus roseus* (L.) G. Don.) using Biochemical Markers

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Abstract

Band designs of esterase (EST), peroxidase (PO) and polyphenol oxidase (PPO) isozymes in several selected cultivars of *Catharanthus roseus* by using native polyacrylamide gel electrophoresis (PAGE) were investigated in this study. It was confirmed that cultivar differences in isozyme polymorphism can be revealed by applied electrophoretic patterns. Three isozyme systems produced a total of 16 bands with polymorphism ranged from 66.6-100%. Considering the patterns of isozyme variations in the five cultivars of *Catharanthus roseus*, it is evident that the cultivar 'First kiss coral' displayed crimson red petal with large white eye' displayed demarked profiles of EST, PO and PPO isozymes than other cultivars. This is the first report on isozyme polymorphism in members of the *Cathanarathus roseus* (L.) G. Don.

Keywords: Isozyme, PAGE, UPGMA, *Vinca rosea*

Introduction

Catharanthus roseus (L.) G. Don. is a perennial plant belonging to the family Apocynaceae and is commonly known as the Madagascar periwinkle was originally classified as *Vinca rosea* by Linnaeus later it is renamed as *Catharanthus* roseus based on their morphological features by G. Don. (van der Heijden *et al.*, 2004). Physiologically it produces important antineoplastic alkaloids such as vincristine and vinblastine are mainly present in the leaves and antihypertensive alkaloids (ajmalicine, serpentine and reserpine) are found in roots (Mishra *et al.*, 2001). Leaves of *C. roseus* are used for the treatment of menorrhagia, rheumatism, dyspepsia, indigestion, dysmenorrheal, diabetes, hypertension, cancer, menstrual disorders, skin diseases, bleeding diarrhea and antiviral properties (Fransworth *et al.*, 1968; Holdsworth, 1990; Joy *et al.*, 1998). It is grown as an ornamental plant in gardens and parks for its different coloured flowers and ever blooming nature. Traditionally markers based on morphological differences among individuals have been used to demonstrate the genetic variability, but with the development of electrophoretic techniques, the biochemical analysis become the cheapest and simplest methods that offer sufficient information and serve as a starting point for DNA based studies. Isozymes have played a key role in identifying genotypes and understanding relationships among close individuals. On the other hand isozymes have the advantage that material is processed by an efficient and inexpensive technique without requiring prior knowledge of the genome, easy to isolate, requiring very small amount of isozymes without the need of blotting and radioactive detection and are moderately reproducible. According to the wide literature survey to date on the assessment of genetic diversity of *Catharanthus* species and cultivars were performed by means of secondary metabolites, AFLP, ISSR, RAPD and RT-SCAR etc. (Chaudhary *et al.*, 2012; El-Domyati et al., 2012; Idrees *et al.*, 2010; Sevestre-Rigouzzo *et al.*, 1993; Shaw *et al.*, 2009). Yet, there has been no previous report on the use of isozyme profiles to characterize the genetic diversity of highly medicinal plant *Catharanthus roseus* cultivars. Hence, the present study was to investigate the intravarietal relationships by three isozyme profiles (esterase, peroxidase and polyphenol oxidase) from 5 different cultivars of *Catharanthus roseus* (L.) G. Don.

Materials and methods

Plant materials

Five cultivars of *Catharanthus roseus* (Fig. 1) viz., i) 'Patricia' white possessing white petal with small yellow center (a), ii) 'Cooler orchid' consisting pinkish red petal with white eye with yellow (b), iii) 'Cooler peppermint' improved shows white petal with small red eye with yellow centre (c), iv) 'First kiss coral' displayed crimson red petal with large white eye (d) and v) Experimental rose pink with eyes represented pale pink petal with radiating red eye (e) were collected from the Herbal Garden, Department of Botany, Andhra University, Visakhapatnam, India.

Received 06 Octombrie 2013; accepted 09 November 2013

Fig. 1. Photographs of flowers of the five cultivars of *Catharanthus roseus* (L.) G. Don.: a) 'Patricia' white possessing white petal with small yellow center; b) 'Cooler orchid' consisting pinkish red petal with white eye with yellow; c) 'Cooler peppermint' improved shows white petal with small red eye with yellow centre; d) 'First kiss coral' displayed crimson red petal with large white eye and e) Experimental rose pink with eyes represented pale pink petal with radiating red eye

Isozyme analysis

Three young leaves were taken in an ice bucket from 3 plants per cultivar. A mixed sample of all leaves per cultivar was used for analysis.

Extraction of isozymes

One gram of leaves from each sample was weighed. The leaves were gently homogenized with the extraction buffer (0.2 M phoshate buffer (pH 7.5)+0.1% PVP+0.1% BSA+10 mM MgCl2+14 mM β-mercaptoethanol) using mortar and pestle at 4 °C. The extracts were centrifuged at 15000 rpm for 10 minutes at 4 °C using refrigerated centrifuge. 500 µl of the supernatant was then mixed with 250 µl of V/V glycerol and bromophenol blue (0.05 mg/ml) was added to the extract. The isozymes were extracted at three different times to test the repeatability and reliability of the biochemical marker.

Electrophoresis

The enzymes were resolved on 12% separating gel and 5% stacking gel polyacrylamide slabs using the electrophoretic systems of Davis (1964). Gels were allowed to run initially with 25-30 mA for 30 min. the current was later increased to 50 mA until the bromophenol blue reached the gel end and all the operations carried out at temperature 4 ^{0}C. After electrophoresis, the gels were carefully removed from the apparatus and carefully washed with electrophoretic buffer and then the gels were incubated to the respective staining solutions for few minutes till the clear bands appeared.

Enzyme staining

Esterase (EST)

The esterase isozymes were visualized by 40mg of 1-naphthylacetate and 40 mg of 2-naphthylacetate were dissolved together and also separately in 1 6ml of 50% (v/v) acetone and mixed with 100 ml of 50 mMTris/HCl buffer (pH 7.1). The gels were incubated for 30 min in this solution, rinsed with tap water and stained 10 to 20 min in 0.2% Fast Blue RR salt solution. The Fast Blue RR salt was dissolved in an appropriate volume of absolute methanol and filtered into 50 mMTris/HCl buffer (pH 7.1) (Balen *et al.*, 2004).

Peroxidase (PO)

For visualization of peroxidase isozymes, 500 mg of benzidine dissolved in 0.5 ml of ethanol and 5 ml of acetic acid and 95 ml of water were added to it. The contents were mixed thoroughly and filtered through cotton. Then 250 µl H_2O_2 was added to this just before staining (Vallejos, 1983).

Polyphenol oxidase (PPO)

The staining solution for staining of polyphenol oxidase isozymes was prepared by dissolving 0.03 M catechol containing 0.05 per cent p-phenylene diamine in phosphate citrate buffer (pH 6.0) (Vallejos, 1983).

Staining reactions were stopped by washing the gels 3-5 times with H_2O and the gels were fixed with 7% acetic acid solution for analysis.

Photographs

Photographs of the gels were taken after staining the gels and were being laid directly on to an illuminator with an opal white screen (avoiding trapped air bubbles) and kept wet during photography by addition of 7% acetic acid with a Cannon camera.

Data analysis

Relative mobility (Rm) values were calculated for each band based on the migration of the band relative to the front or tracking dye. The gels were scored as presence (+) or absence (-) of isozyme bands. The produced clear well defined bands are used to estimate the levels of polymorphism by dividing the polymorphic bands by the total number of shared bands. Depending upon the presence or absence of bands similarity indices (SI) were calculated (Nei and Li, 1979). Cluster analysis UPGMA (unweighted pair group method with arithmetic averages) was performed on the similarity index by using statistical software SPSS for windows package (Version 10).

Results and Discussion

Three isozyme systems including esterase (EST), peroxidase (PO) and polyphenol oxidase (PPO) were used to test the genetic diversity among the five different cultivars of *Catharanthus roseus* (L.) G. Don. Based on the isozyme patterns of these 3 enzyme systems the investigated 5 cultivars were identifiable (Tab. 1 and Fig. 2).

Esterase (EST)

A total of 5 esterase bands (Rm value ranged from 0.050-0.575) of which 80% polymorphism was recorded between the cultivars (Tab. 2). Cultivars 'a', 'b' and 'c' giv-

ing the highest number of bands (4), while the other cultivars 'd' and 'e' expressed three bands each.

Five zones of peroxidase isozymes (PO 1-PO 5) with Rm value (0.050-0.600) were scored in the five cultivars. Four cultivars i.e., ('a', 'b', 'c' and 'e') expressed maximum 3 bands each and the cultivar 'd' expressed only two bands. Interestingly this isozyme system represented 100% polymorphism between the cultivars (Tab. 2).

Tab. 1. Presence (+)/absence (-) data matrix of the isozymatic study

S.No.	Type of Isozyme (Rm Value)	*Catharanthus roseus* cultivars 'a'	'b'	'c'	'd'	'e'
1.	Esterase isoforms (EST)					
	EST 1(0.050)	+	+	+	+	+
	EST 2(0.075)	-	-	+	-	-
	EST 3(0.112)	+	+	-	+	+
	EST 4(0.175)	+	+	+	+	-
	EST 5(0.575)	+	+	+	-	+
2	Peroxidase isoforms (PO)					
	PO 1(0.050)	+	-	+	+	-
	PO 2(0.075)	-	+	-	-	+
	PO 3(0.200)	+	-	+	+	+
	PO 4(0.225)	-	+	-	-	-
	PO 5(0.600)	+	+	+	-	+
3	Polyphenol oxidase isoforms (PPO)					
	PPO 1(0.025)	+	+	+	+	+
	PPO 2(0.075)	-	+		-	-
	PPO 3(0.112)	-	-	+	-	-
	PPO 4(0.225)	+	+	+	+	+
	PPO 5(0.625)	-	-	-	+	-
	PPO 6(0.650)	+	+	+	-	+

a – e: refer to the materials.

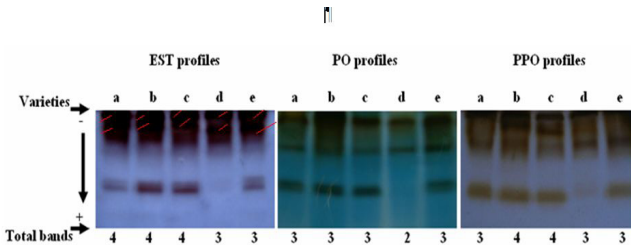

Fig. 2. Different isozyme (EST, PO and PPO) profiles in 5 different cultivars of *Catharathus roseus* (L.) G. Don. Bars distinguish the closely separated bands

Polyphenol oxidase (PPO)

Different banding patterns were observed for polyphenol oxidase (PPO) isozyme. As a whole a total of six different bands (PPO 1-PPO 6) with Rm 0.025-0.650, cultivars 'b'&'c' expressed maximum bands (4), on the other hand this enzyme exhibited very low polymorphism (66.6%) when compared to EST and PO (Tab. 2).

Tab. 2. Number and types of isozymes bands as well as the total polymorphism percentages generated in the five different cultivars of *Catharanthus roseus* (L.) G. Don

Isozyme system	Monomorphic bands	Polymorphic bands		Total bands	Polymorphism (%)
		Unique	Shared		
EST	1	1	3	5	80.0
PO	0	1	4	5	100.0
PPO	2	3	1	6	66.6

Similarity index (SI) for five cultivars of Catharanthus roseus ranged from 52.63 to 84.21%. Highest per cent similarity (84.21) was recorded in 'a'&'e' pair. The dendrogram (UPGMA) resulting from cluster analysis alienated into two groups, group I possessing two cultivars 'a' and 'b' and group II consisting three cultivars 'c', 'd' and 'e' (Fig. 3).

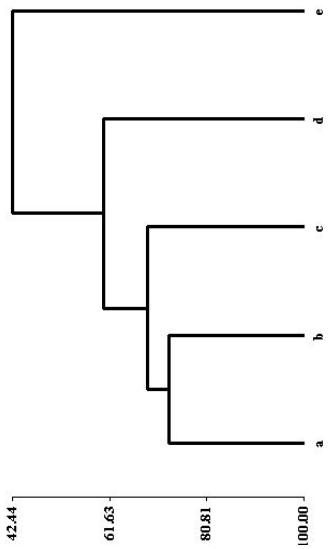

Fig. 3. UPGMA for 5 different cultivars of *Catharanthus roseus* (L.) G. Don. based on SI of three isozymes

Polymorphism is essential in to use of isozymes as genetic marker. When considered the EST, PO and PPO isozymes polymorphism and their coding capacity of the molecules with different velocity, they are convenient for identification of genotypical differences. The topology of the plant based on the isozyme data in addition to such large number of polymorphic zones reflect the validity of the isozyme data to study the genetic diversity and taxonomic relationships at intraspecific levels in Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don.). Such discriminations at the intraspecific levels by isozyme profiles

were previously scored in banana (Mandal *et al.*, 2001) and *Gymnema sylvestre* (Nair and Keshavachandran, 2006). The cultivars 'First kiss coral' displayed crimson red petal with large white eye' expressed special and demarked banding patterns for EST, PO and PPO isozymes, suggests that the cultivar might be a hybrid (Mustafa *et al.*, 2005). This variation may be due to intraspecific natural hybridization and subsequent dispersion of pollen grains and seeds.

In future studies, correlations between isozyme markers and pharmaceutically significant characters, such as vinca alkaloids need to be determined for the expansion of breeding programmes. Further uses of isozymes in a breeding programme, besides characterization and identification of cultivars, include the possibility to determine the pollen parent effect of different *Catharanthus roseus* cultivars during fruit development.

Acknowledgements

One of the authors (O. Aniel Kumar) is grateful to UGC-SAP-CAS-I, Department of Botany, Andhra University, Visakhapatnam for providing financial assistance.

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