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# Genetic diversity and alkaloid production in *Catharanthus roseus*, *C. trichophyllus* and their hybrids

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**Summary**

Inter and intraspecific variation was analyzed in two *Catharanthus* species with regard to isozyme polymorphism and indole alkaloid content in roots and leaves. No significant differences in alkaloid production were observed in three groups of *C. roseus* plants individualized for their flower color. Conversely, comparisons between *C. trichophyllus* and *C. roseus,* showed large differences of alkaloid profiles in both roots and leaves. Specific isozyme markers on four presumed loci were found allowing us to establish that natural hybridizations could occur between the two species when grown together. Experimental hybridizations confirmed that introgressions were feasible but suggested that a reproductive barrier was acting and involved interspecific incompatibility. The identification and assay of the main alkaloid compounds in natural interspecific hybrids displayed such a high hybrid vigor that interspecific hybridization may present a new and successful way of improving alkaloid production in *Catharanthus* species.

## Introduction

The periwinkle plant *Catharanthus roseus* (L.) G. Don originating from Madagascar was introduced as an ornamental plant in various tropical and subtropical countries such as the Ivory Coast. This species contains a large variety of alkaloids (Svoboda & Blake, 1975) and some of them are of great interest for medical use: ajmalicine has antihypertensive properties and vincristine and vinblastine are active antitumoral compounds (Conti & Creasey, 1975). Nevertheless, as vincristine and vinblastine are only present in trace amounts in the leaves, the preparation of these alkaloids has, for economic purposes, still to rely on the extraction of their monoindole precursors, catharanthine and vindoline, which are synthesized in more substantial quantities (Farnsworth, 1961) and which can yield other potent compounds by hemisynthesis. Alkaloids are accumulated in all the tissues but some of them seem to be organ-specific: ajmalicine and serpentine are essentially present in the roots while catharanthine and vindoline are accumulated in aerial parts (Endo et al., 1987; Balsevitch et al., 1988), some of the enzymes involved in the last steps of indole alkaloid biosynthesis being located in the chloroplasts (Luca & Cuttler, 1987) or being light-stimulated (Luca et al., 1988). Moreover, a large variation in alkaloid production is also reported between individuals (Levy et al., 1983; Weissenberg et

al., 1988) and differs according to growing conditions (Daddona et al., 1976) and culture stages (Reda, 1978).

Among the seven *Catharanthus* species described by Markgraf (1976), *C. roseus* is the most intensively studied in terms of analyses of alkaloid content; horticultural types based on flower colors in *C. roseus* have been evaluated in this respect (Patra, 1982; Levy et al., 1983). Nevertheless, little is known about the taxonomic status and the genetic diversity between species in relation to the alkaloid content even though spontaneous hybridizations have been reported between some species of *Catharanthus* (Veyret, 1974; Plaizier, 1981). The aim of this paper is to assess and compare at the isozyme and alkaloid levels the genetic diversity of *C. roseus* and *C. trichophyllus* (Baker) Pichon which is another species considered to possess interesting characteristics. On the other hand, the occurrence and the identification of spontaneous hybrids in our material provided an opportunity to investigate the basis of interspecific hybridizations and breeding procedures in terms of alkaloid production.

## Material and methods

Two strains of *C. roseus* and *C. trichophyllus* were obtained from the Research Department of Pierre Fabre Médicaments (Castres, France). Before evaluation, the two strains were maintained in field conditions for seed multiplication and were grown for 2 cycles at ORSTOM in Ivory Coast (Research Station of Adiopodoumé). At maturation, pods were randomly collected on 100 plants for each species.

The experimental studies were started after this preliminary multiplication and two plots were grown separately; 36 plants for each species were characterized 130 days after sowing to confirm botanical traits and growing habit. The biomass production of each individual was determined by measuring fresh and dried weights (oven dried at 50°C for 4 days) of the leaves and roots. The plants were scored for isozyme polymorphism by starch gel electrophoresis as described previously by Second and Trouslot (1980): 8 different enzymes were assayed but only 4 displayed clear-cut patterns and were kept for analysis: malate dehydrogenase (*Mdh*), glutamate oxaloacetate transaminase (*Got*), esterase (*Est*), phosphoglucoisomerase (*Pgi*); in addition, 23 intermediate plants only found in the *C. trichophyllus* plot were included in the isozyme analysis since owing to phenotypic traits, these plants were suspected of being hybrid derivatives.

Controlled hybridizations were performed in a

*Table 1.* Botanical characteristics and evaluation of some traits involved in biomass production in two species of *Catharanthus* and their interspecific hybrids

| | *C. roseus* | Hybrid derivatives | *C. trichophyllus* |
|---|---|---|---|
| Vegetation | Several stems with low branching | Erect stems with circular section | Erect stems with quadrangular section<br>Single stem with high branching |
| Leaves | Oblong and petioled | Ovate slightly petioled | Ovate to lanceolate and subsessile |
| Fruit shape | Erect | Curved | Curved |
| Flower color | Pink, white or white red-eyed corolla | Purple corolla with dark or yellow eye | Purple yellow-eyed corolla |
| Sepal length (mm) | 4 | 7 | 7 |
| Length of corolla tube (mm) | 25 | 25 | 22 |
| Petal length (mm) | 12 | 12 | 10 |
| Plant height (cm) | 66 | 80 | 82 |
| Main root length (cm) | 7 | 7 | 7 |
| N° of ramificat./stem | 9 | 13 | 13 |

greenhouse with some of the previously characterized individuals. *Catharanthus* species are predominantly self-pollinated and need emasculation of the flower buds 2–3 days before anthesis by cutting the corolla tip between stigma and anthers. Fertilization was attempted during the three following days by hand pollination twice a day. Pollinated buds were protected by paper bags to prevent accidental pollination and seed loss at the maturation stage.

Biochemical analyses were carried out in the Plant Physiology Laboratory at ORSTOM in Ivory Coast. Estimation of alkaloid contents was performed on 9 representative individuals in each species and 5 plants of each flower type in *C. roseus*. Alkaloid contents were also determined on seven presumed natural hybrids which were chosen on the basis of their typical hybrid isozyme patterns on the four loci surveyed. For leaf alkaloid extraction, dried and finely powdered leaf samples (1.5g of each plant) were suspended in 70ml methanol/tartaric acid 1% (55/45) for 90min at room temperature. The suspension was filtered and the methanol evaporated. After alkalinisation (pH9–10), the solution was extracted twice with $CHCl_3$ (40ml). The resulting $CHCl_3$ extract was dried over anhydrous $Na_2SO_4$, filtered and concentrated to dryness. For root alkaloid extraction, powdered root samples (1g) were suspended for 90min in 10% acetic acid (70ml) and then extracted as described above. Residues were disolved in methanol (3ml) and the alkaloid content was estimated by reverse-phase liquid chromatography according to the technique previously described (Nef et al., 1991). The retention times for ajmalicine, catharanthine, serpentine and vindoline were 13.3, 15.5, 17.2 and 18.5min respectively in this system.

## Results

### *Phenotypic characterization*

Our observations of the plants agreed with the expected botanical characteristics (Table 1). The sample of *C. roseus* showed three different flower types with pink, white or white red-eyed corollae. These three flower types are commonly observed in mixtures in *C. roseus* strains but they were not associated with other phenotypic characters in our strain and did not require a special taxonomic status as previously reported by Markgraf (1976). *C. trichophyllus* was more vigorous and showed more branching, the leaves were slightly lanceolated and all the flowers were red-purple with a yellow eye. In the *C. trichophyllus* plot, some plants were considered separately because they presented intermediate characteristics for leaves, pod shape and corolla color (Table 1). These plants were vigorous with high flowering and seed set ability and appeared globally closer to *C. trichophyllus* than to *C. roseus*. Fresh and dried weights at the full flowering-early maturation stage were significantly higher in *C. trichophyllus* than in *C. roseus* for both leaves and roots, while the off-types were close to *C. trichophyllus* (Table 2).

*Table 2.* Fresh and dry weights (g per plant) of leaves and roots in two *Catharanthus* species and in natural interspecific hybrids

| | Number of individuals | Leaves | | | | Roots | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | fresh weight | | dry weight | | fresh weight | | dry weight | |
| | | m | sd | m | sd | m | sd | m | sd |
| *C. roseus* | | | | | | | | | |
| mixed flower colors | 36 | 177 | 105 | 25 | 12 | 20 | 8.5 | 5 | 2.0 |
| pink | 5 | 146 | 35 | 28 | 8 | 19 | 6.3 | 5 | 1.5 |
| white | 5 | 153 | 90 | 23 | 15 | 18 | 7.1 | 5 | 2.0 |
| white red-eyed | 5 | 192 | 57 | 28 | 7 | 22 | 5.0 | 6 | 1.4 |
| *C. trichophyllus* | 36 | 202 | 93 | 41 | 26 | 36 | 14.0 | 10 | 3.9 |
| Interspecific hybrids | 7 | 248 | 93 | 50 | 25 | 40 | 8.4 | 11 | 3.2 |

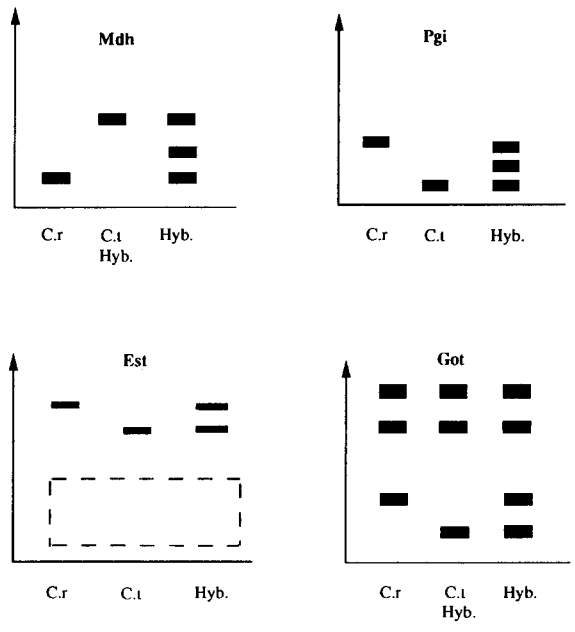


*Fig. 1.* Isozyme patterns of four enzymes observed in *Catharanthus roseus* (C.r.), (n = 36), *C. trichophyllus* (C.t.), (n = 36) and in natural hybrids (Hyb.), (n = 23). malate dehydrogenase (Mdh), phosphoglucoisomerase (Pgi), Glutamate oxaloacetate transaminase (Got), esterase (Est).

### *Isozyme polymorphism*

Isozyme polymorphism was low and demonstrated similar profiles within and between species for most of the different enzymes assayed. Nevertheless, four enzymes allowed identification of specific electromorphs for each species while the intermediate plants produced either the pattern found in *C. trichophyllus* or a hybrid pattern according to the enzyme multimeric structure (Fig. 1). Thus, *Pgi* and *Mdh* zymograms showed a single band with different mobility for each species while the off-types most often exhibited a typical three band hybrid pattern for a dimeric enzyme with codominant alleles. With *Got* and *Est,* several loci are usually observed in plants, but only the slowest band for *Got* and a fast band with low intensity for *Est* showed a different mobility between the two species; the intermediate plants mainly presented two bands corresponding to a locus encoding for a monomeric enzyme. Thus, we identified 4 diagnostic loci between the two species since no variation was scored within each species and the off-types can be assumed to come from natural hybridizations between *C. roseus* and *C. trichophyllus.*

In order to refine the study of natural introgressions between *Catharanthus* species, multilocus patterns of hybrid derivatives were analyzed taking into account the number of alleles coming from *C. trichophyllus* (T) and the number of heterozygous loci (H). T and H could range from 0 to 8 and from 0 to 4 respectively and, assuming that the isozyme loci were independent of each other, these two combined parameters allowed the expected distribu-

*Table 3.* Comparison of distribution of natural interspecific hybrids between *C. roseus* and *C. trichophyllus* according to T and H parameters with theoretical expectations in $F_2$ and in back-cross with *C. trichophyllus*

| T (1) | H (2) | Theoretical distribution | | Number of individuals |
|---|---|---|---|---|
| | | $F_2$ | Back cross on *C. trichophyllus* | |
| 8 | 0 | 0.004 | 0.062 | 0 |
| 7 | 1 | 0.032 | 0.250 | 0 |
| 6 | 2 | 0.096 | 0.375 | 2 |
| 6 | 0 | 0.016 | 0 | 0 |
| 5 | 3 | 0.126 | 0.250 | 8 |
| 5 | 1 | 0.096 | 0 | 0 |
| 4 | 4 | 0.062 | 0.062 | 13 |
| Pooled other combinations with T <4 | | 0.574 | 0 | 0 |

(1).(2): number of alleles coming from *C. trichophyllus* (T) and number of heterozygous loci (H) considered on the four loci: *Est. Got. Pgi. Mdh.*

tions to be distinguished in selfed or in back-cross progeny. Most of the off-types followed a genic structure of $F_1$ hybrid with four loci in a heterozygous state while the other plants corresponded to progeny arising from $F_2$ or from back-cross with *C. trichophyllus*(Table 3). The offspring number was not large enough to separate $F_2$ from back cross segregations with assurance but the observed distribution was truncated and suggested that these hybrid derivatives included, in addition to true $F_1$ hybrids, a major component of plants coming from back-cross with *C. trichophyllus*; this was consistent with the presence of off-types which was limited to the *C. trichophyllus* plot.

### *Interspecific hybridization*

Controlled hybridizations were made to measure the direction of gene flow between the two species using plants previously evaluated for isozyme polymorphism. As already noted by Parker (1980), crosses were successful with high seed set and good germinating ability of $F_1$ when *C. trichophyllus* was the female parent (Table 4). Conversely, reciprocal crosses failed to form pods in all cases; these observations, coupled with the informations relating the genetic structure of natural hybrid derivatives support an interspecific incompatibility model between *C. roseus* and *C. trichophyllus*; this incompatibility was assumed previously to have a gametophytic genetic basis (Parker & Vitti, 1986) and consequently acted as a restriction of potential introgressions from *C. trichophyllus* towards *C. roseus*.

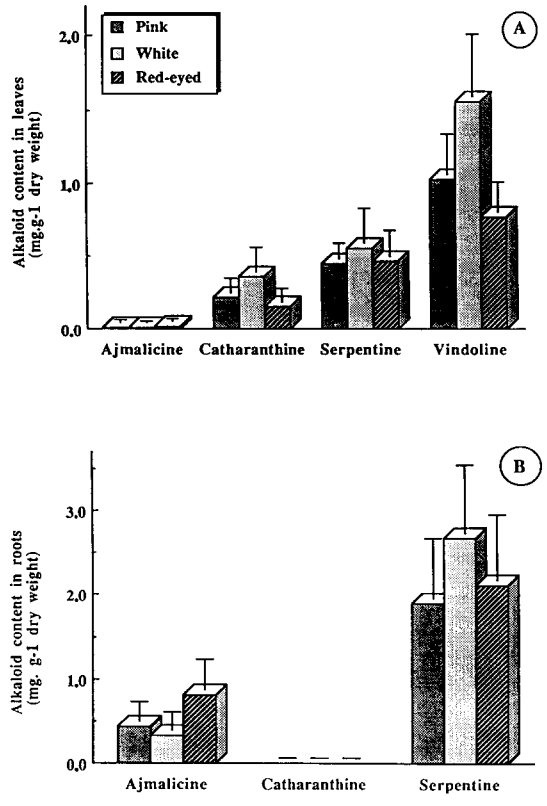


*Fig. 2.* Alkaloid contents of leaves (2A) and roots (2B) in 3 samples of *C. roseus* selected for flower color.

### *Alkaloid production*

HPLC determination of the main alkaloid compounds was performed firstly on the three subsamples of *C. roseus* isolated according to their flower color. As observed in Fig. 2, no qualitative differences were seen between the pink, white and red-eyed flower plants. Vindoline in the leaves (Fig.

*Table 4.* Results of interspecific hybridizations between *C. roseus* and *C. trichophyllus*

| Crosses (female× male) | Crosses | | $F_1$ seed set | | Germination % |
|---|---|---|---|---|---|
| | N° of pollinated flowers | N° of harvested pods | N° of $F_1$ seeds | Crossability % | |
| *C. roseus*× *C. trichophyllus* | 15 | 0 | 0 | – | – |
| *C. trichophyllus*× *C. roseus* | 12 | 12 | 190 | 79 | 38 |
| Control | Emasculated | | | | |
| *C. roseus* | 6 | 0 | | | |
| *C. trichophyllus* | 8 | 0 | | | |

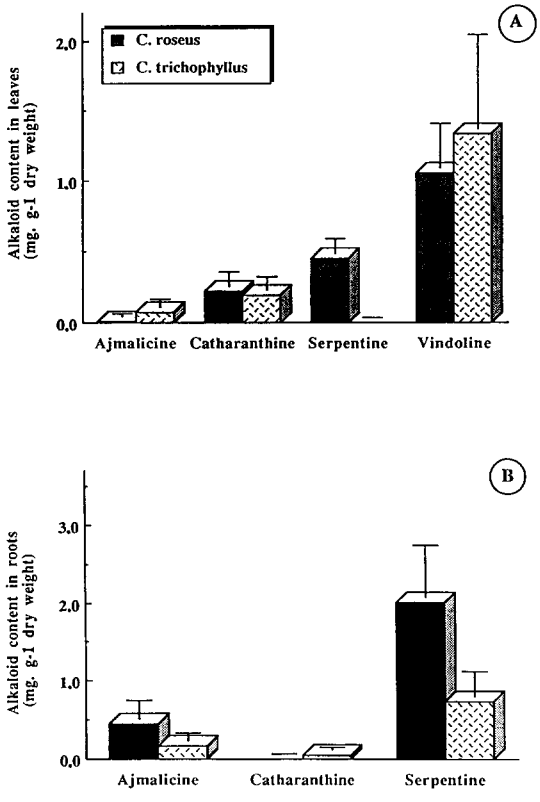


*Fig. 3.* Alkaloid contents of leaves (3A) and roots (3B) in *C. roseus* and *C. trichophyllus.*

2A) and serpentine in the roots (Fig. 2B) were the most abundant compounds whatever the flower color. Ajmalicine was present in large amounts in the roots but only at trace levels in the leaves while catharanthine and vindoline levels were not detected in roots. Moreover, from the quantitative point of view, the three subsamples were not significantly different even if the white colored individuals showed a tendency to be more productive.

Conversely, comparisons between the two species showed major qualitative and quantitative variations for the same growing stage. Alkaloid profiles differed in *C. trichophyllus* as evidenced by the absence of serpentine in leaf extracts (Fig. 3A) and by the production of catharanthine in roots (Fig. 3B). Except for ajmaciline, no significant differences of alkaloid contents in leaves were found between the two species. In roots, great quantitative differences appeared and the content of each alkaloid compound was significantly different between the two species ($P = 0.01$), *C. roseus* being 2.6 fold more productive in ajmalicine and serpentine than *C. trichophyllus.*

In the hybrids, the alkaloid content appeared to be predominantly concentrated in the leaves in a similar manner to *C. trichophyllus* and all the organ-specific alkaloids found in each parent were also observed in the hybrids (Table 5): hybrid leaves contained serpentine like *C. roseus* leaves and their roots accumulated catharanthine like *C. trichophyllus* roots. A negative correlation between the ajmalicine and serpentine contents was observed among hybrid individuals while no such opposition was found between vindoline and catharanthine productions which both increased. The alkaloid production of the hybrids was compared to the parents using classical formulas of heterosis estimation: firstly, in comparing the mean values of the hybrids with the best parent and secondly, in taking as reference the best individual values for each alkaloid (Table 5). Except for serpentine in leaves and ajmalicine in roots, the production of the other alkaloids was significantly higher than the best parent. The differences remained high when the best hybrid for the overall alkaloid production of the four substances considered was compared with the best individual values among the parents.

## Discussion

Natural interspecific hybridizations of periwinkles have been observed in Madagascar when the different species are grown together in botanical gardens or when the geographical distribution overlaps (Veyret, 1974; Markgraf, 1976); most of these hybridizations concerned *C. longifolius*× *C. roseus.* Although *Catharanthus* species are predominantly autogamous, low rates of outcrossing in *C. roseus* have been measured using markers involved in seedling pigmentation and flower color (Khrishnan et al., 1979). Outcrossing levels may be variable following environmental conditions and pollinator effects; this suggests that the flowering biology can maintain an efficient sexual isolation in the natural conditions of Madagascar but that conditions in Ivory Coast allow a significant outcrossing rate and

introgression opportunities. Nevertheless, a reproductive barrier is considered to act against *C. trichophyllus* since no evidence of introgressions were found from *C. trichophyllus* to *C. roseus* after two multiplication cycles without control of fecondation. *C. roseus* is considered as the only *Catharanthus* species which presents incompatibility against the others (Plaizier, 1981). Our experimental hybridizations confirm this and suggest a genetic model similar to that between the cultivated tomato *Lycopersicon esculentum* and wild species where $F_1$ hybridizations and backcrosses are only feasible in one direction (McGuire & Rick, 1954).

The study of the alkaloid production in leaves and roots of the two *Catharanthus* species demonstrated that the alkaloid profiles were species-specific with slight quantitative variations within each species. Differences of alkaloid amounts do not correspond to different flower types in *C. roseus* and do not confirm previous reports stating that certain flower types could be more interesting for their alkaloid content (Patra, 1982). Nevertheless, this lack of significant differences can arise from the heterogeneity of our material which was not grown as pure inbred lines and from the culture stage. At the interspecific level, further studies on more representative samples should be undertaken to confirm that some compounds can be considered as specific markers in the same way as isozyme markers.

Indole alkaloid compounds come from tryptophan metabolism and are related in complex metabolic pathways which are not yet clearly elucidated. Ajmalicine and serpentine come from the same side branch of the main pathway leading to vindoline, catharanthine and the dimeric antineoplastic compounds, vincristine and vinblastine (Noe et al., 1984; Merillon et al., 1986; Doireau et al., 1987). Ajmalicine is converted into serpentine through the activity of vacuolar basic peroxidases (Blom et al., 1991) and this conversion seems to be light-dependent (Knobloch et al., 1982). Our results suggest that the enzymatic systems leading to the alkaloid production were differently regulated in the two species of *Catharanthus*. In hybrids, new equilibrium states seem to be obtained between the different metabolic pathways that allow the occurrence of all

*Table 5.* Alkaloid production (mg·g-1 dry weight) in interspecific hybrids between *C. roseus* and *C. trichophyllus* and estimation of heterosis in percent of the better parent (% BP)

| | Alkaloid production | | | Heterosis estimation (% BP) | |
|---|---|---|---|---|---|
| | Hybrids | | better individual among parents | mean values (1) | best values (2) |
| | m | sd | | | |
| Leaves | | | | | |
| Ajmalicine | 0.14 | 0.11 | 0.16 (C.t.) | 197** | 162 |
| Catharanthine | 1.04 | 0.71 | 0.36 (C.r.) | 467** | 410 |
| Serpentine | 0.40 | 0.42 | 0.58 (C.r.) | 87 | 138 |
| Vindoline | 3.30 | 1.23 | 2.30 (C.t.) | 246** | 205 |
| Total | 4.88 | 1.93 | 2.73 (C.t.) | 280** | 266 |
| Roots | | | | | |
| Ajmalicine | 0.25 | 0.20 | 0.81 (C.r.) | 56* | 66 |
| Catharanthine | 0.20 | 0.17 | 0.09 (C.t.) | 410** | 608 |
| Serpentine | 2.22 | 0.91 | 3.19 (C.r.) | 110* | 68 |
| Total | 2.67 | 0.96 | 4.10 (C.r.) | 109 | 125 |

*, **significant at $P < 0.05$ and $P < 0.01$, respectively.

(1): Heterosis expressed in comparing the mean values of hybrids and the better parent.

(2): Comparison between the best hybrid for the overall alkaloid production and the best individual value among parents.

(C.r.): *C. roseus.*

(C.t.): *C. trichophyllus.*

the alkaloid compounds at the same time. The better production of hybrids can come from complementary effects between genes involved in enzymes of the alkaloid pathways and can suggest heterosis phenomena in secondary metabolism although evidences at molecular level are not yet available to confirm it.

In hybrids, the new equilibria of metabolic pathways can explain the larger variation between individuals for each alkaloid taken separately; nevertheless, overall alkaloid production in hybrids is more stable and shows reduced variance when compared to the parents even if this production is generally considered to be predominantly environment-dependent. Thus, it is possible to obtain single individuals which show a well balanced alkaloid production. If some intraspecific variation and heterosis can be utilized with *C. roseus* (Levy et al., 1983), a more substantial genetic improvement can be expected in exploiting heterosis between *C. roseus* and *C. trichophyllus*. On the other hand, *in vitro* micropropagation can be performed successfully with *C. roseus* and clones display reduced variance for alkaloid contents after growing in field conditions when compared to seed-borne plants (unpublished data). This indicates that environmental effects are not so strong and that breeding for hybrids having high alkaloid contents, coupled to clonal propagation, can provide an original means of improving alkaloid production in *Catharanthus*.

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