ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization (2016) **14**(4); 283–302
doi:10.1017/S1479262116000150

Breeding medicinal plant, periwinkle [*Catharanthus roseus* (L) G. Don]: a review

R. N. Kulkarni[1]*†, K. Baskaran[1] and Tripta Jhang[2]

[1]*CSIR – Central Institute of Medicinal Plants (CIMAP) Research Centre, Bangalore 560 065, Karnataka, India and* [2]*Genetics & Plant Breeding Division, CSIR–CIMAP, Lucknow 226 015, UP, India*

Received 1 April 2016; Accepted 1 April 2016 – First published online 2 May 2016

Abstract

Periwinkle [*Catharanthus roseus* (L) G. Don] has become one of the very extensively investigated medicinal plants after the discovery of two powerful anti-cancer alkaloids, vinblastine and vincristine, in its leaves more than 50 years ago. These alkaloidal drugs are still in clinical use. Also, periwinkle is still the only source of these alkaloids and their precursors, catharanthine and vindoline. Low concentrations of these alkaloids in the plant and, therefore, high costs of their extraction have led to tremendous efforts towards understanding their biosynthesis and exploration of alternate ways of their production such as, chemical synthesis, cell, tissue and hairy root cultures, and metabolic engineering of heterologous organisms. Literature on this plant is quite voluminous, with an average of about 80 publications per year during last three decades (1985–2015). Nearly 60% of these publications are on physiology, biochemistry, cell and tissue culture, phytochemistry, metabolic and genetic engineering aspects. In spite of these efforts, an economically viable alternative to field-grown periwinkle plants as a source of these alkaloids has not yet been found. Biosynthesis of *C. roseus* alkaloids is a complex process involving many genes, enzymes, regulators, inter- and intracellular transporters, cell types, organelles and tissues and its current understanding is still considered to be incomplete to produce *C. roseus* alkaloids through metabolic engineering/synthetic biology. Till such time, breeding periwinkle varieties with higher concentrations of anti-cancer alkaloids for cultivation can be an alternate approach to meet the demand for these alkaloids and reduce their costs. While literature on cell and tissue culture, phytochemistry, metabolic and genetic engineering aspects of periwinkle has been reviewed periodically, crop production and plant breeding aspects have received little attention. In this paper, an attempt has been made to bring together published information on genetics and breeding of periwinkle as a medicinal plant. Some probable constraints which may have hindered taking up periwinkle breeding are identified. Initially, quite a few attempts have been made at genetic improvement of periwinkle through induced polyploidy, and subsequently through induced mutagenesis. Mutations, both natural and induced, provide a valuable resource for use in breeding and in functional and reverse genomics research. It is only during last 6–7 years, genetic diversity has been assessed using molecular markers and very recently molecular markers have been identified for marker-assisted selection for alkaloid yield.

Keywords: anti-cancer alkaloids, autotetraploids, breeding behaviour, cleistogamy, cytology, induced mutants, inheritance, marker-assisted selection, secondary metabolism

This article is respectfully dedicated to Professor V. L. Chopra on the occasion of his 80th Birthday.

*Corresponding author.
E-mail: krnpbg@yahoo.co.in
†Present address: G1, Prashanth Apts., 5th Cross, Ganganagar, Bangalore 560 032, Karnataka, India.

Introduction

The history of use of plants for therapeutic purposes is perhaps as old as the history of human civilization on this planet. Earliest records of the usage of plants date back to 2600–1550 BC in ancient Greece, Egypt and India (Salim *et al.*, 2008; Dias *et al.*, 2012). Out of about 422,000 plant species estimated to be on this planet, about 52,800 are estimated to be medicinal plants species, which, however, are not distributed evenly over all plant families; some plant families like Apocynaceae, Araliaceae, Apiaceae, Asclepiadaceae, Canellaceae, Guttiferae and Menispermaceae have higher proportion of medicinal plant species than others (Schippmann *et al.*, 2003). A total of 163 drugs have been discovered from 114 plant species so far (Lahlou, 2013), out of which 122 have been discovered in 94 plant species based on their ethno-medical uses (Fabricant and Farnsworth, 2001). The discovery of anti-cancer alkaloids in leaves of Madagascar periwinkle [*Catharanthus roseus* (L) G. Don], an Apocynaceous plant, is considered to be one of the most significant discoveries of drugs made so far from higher plants, which have had their therapeutic efficacy and utility proven beyond doubt (Tyler, 1988; Pezzuto, 1997).

The medicinal property of periwinkle has been recorded in folklores and traditional medicine literature as early as in 50 B.C. (Husain, 1993). Different parts of this plant have been used in various forms in traditional and/home remedies all over the world for the treatment of a wide range of ailments such as diabetes, fevers, malaria, menorrhagia, hypertension, cancer, stomach ailments, heart disease, leishmaniasis, amenorrhea, dysmenorrhoea, rheumatism, liver disease, etc., (Ross, 1999). The wide spectrum of pharmaceutical properties of this plant caught the interest of the scientific world.

During the mid-1950s, two independent research groups, one at the University of Western Ontario, Canada, and the other at Eli Lilly Company, Indiana, USA, investigating the reported folkloric use of periwinkle as an oral hypoglycaemic agent could not demonstrate hypoglycaemia in either normal or experimentally induced hyperglycaemic rabbits. They observed that extracts of periwinkle leaves produced leukopenic activity in rats and prolongation of life of DBA/2 mice infected with P1534 leukaemia, respectively (Svoboda, 1975). Further phytochemical investigations led to the discovery of two alkaloids, vincaleukoblastine [vinblastine (VLB)] by Noble *et al.* (1958) and leurocristine [vincristine (VCR)] by Eli Lilly Company, USA, possessing anti-cancer property (Svoboda, 1975; Tyler, 1988). Interestingly, VLB and VCR were discovered in samples collected from Jamaica and Philippines although periwinkle is endemic to Madagascar (Cragg and Newman, 2005). Four alkaloids from periwinkle are used clinically: VLB, vinorelbine (VRL), VCR and vindesine (VDS). VLB sulphate, commercially known as Velban® is used in the treatment of Hodgkin's disease, lymphosarcoma, neuroblastoma and choriocarcinoma. VCR sulphate, commercially known as Oncovin® or Vincovin® is used in the treatment of leukaemia in children and reticulum cell sarcoma (Svoboda and Blake, 1975). Two semi-synthetic bisindole alkaloids, VRL, marketed as Navelbine® and VDS marketed as Eldisine® and Fildesin® are used in the treatment of breast cancer and bronchial cancer, and acute lymphoblastic leukaemia and refractory lymphoma, respectively (Bruneton, 1995; Pezzuto, 1997). These alkaloids are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers (Cragg and Newman, 2005). In 2005, the market for VCR and VLB was estimated at US$150–300 million and Oncovin® and Velban®, are sold for a total of US$100 million per year (Schmelzer, 2007).

The discovery of ajmalicine, used in the treatment of circulatory diseases, especially hypertension, in roots of periwinkle followed their detection as an adulterant in *Rauvolfia* roots exported from India. Since it proved as valuable source as *Rauvolfia* of ajmalicine, it was readily accepted as an alternate source of this alkaloid (Krishnan, 1995; Singh, 1996).

Although anti-cancer alkaloids VLB and VCR were discovered more than 50 years ago, periwinkle still remains the sole source of these alkaloids. Apart from VLB and VCR, periwinkle produces more than 130 diverse groups of alkaloids (Zhu *et al.*, 2014) and has been termed as an alkaloid engine (Dugo de Bernonville *et al.*, 2015). However, the low contents of VLB and VCR (1 g and 20 mg in 1000 kg of plant material, respectively) in the plant (Tyler, 1988) and high costs of their extraction have led to extensive efforts towards increasing their production and reducing their costs through various approaches. As a result, periwinkle has emerged as one of the most extensively investigated medicinal plants and is regarded as model 'non-model' plant for the study of alkaloid metabolism in plants (Facchini and De Luca, 2008). Around 3000 scientific publications have appeared on this plant during last 50 years (1963–2015) with an average of about 58 publications per year. About 80 papers have been published every year during the last three decades suggesting continued interest in this plant. About 67% of these papers are in the areas of biochemistry and physiology, cell and tissue culture, molecular biology and genetic engineering, and phytochemistry. The remaining 33% of the publications are in the areas of botany, crop production and management, plant pathology, pharmacology, entomology and nematology, genetics and plant breeding (Fig. 1). While during early years (1963–1974) after the discovery of

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

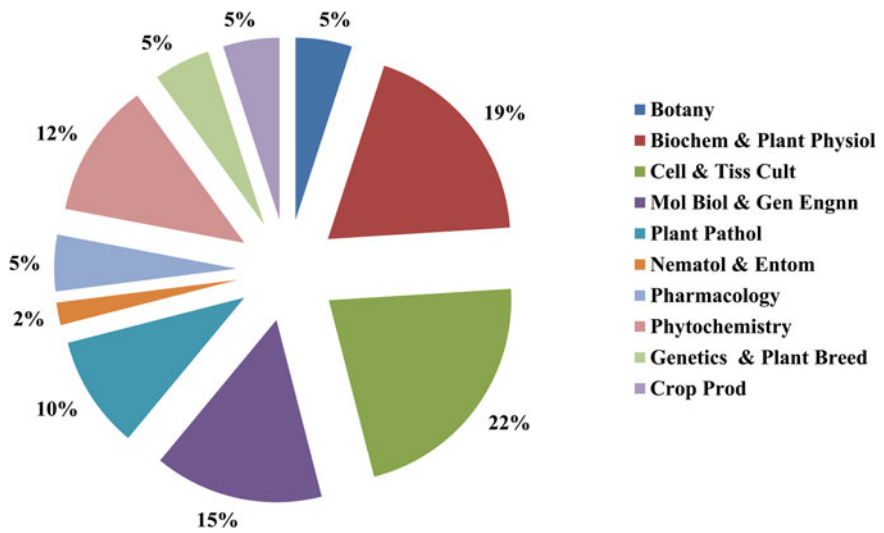

Fig. 1. Distribution (%) of publications on periwinkle in different subject areas during the period 1963–2015 (search with key words *C. roseus* or *V. roseus* at websites http://newcrops.com.au/; http://www.ncbi.nlm.nih.gov/pubmed and https://scifinder.cas.org/scifinder).

these alkaloids, botany and phytochemistry were the top two researched areas; biochemistry and physiology, cell and tissue culture emerged as the two most researched areas during the next two decades, i.e. 1975–1984 and 1985–1994. However, during the last two decades, the highest numbers of publications have appeared in the area of molecular biology and genetic engineering (Fig. 2). This is in conformity with the general trend observed in the most actively pursued areas of plant sciences during these five decades. Thus, it is apparent that although periwinkle can be easily cultivated in tropical regions, relatively very little attention has been paid to field production of this plant as a source of these important alkaloids as compared with their production *in vitro*. This may be because cancer is regarded as afflicting mainly developed countries (Cragg and Newman, 2005), i.e. mostly in temperate regions, where periwinkle does not thrive well. Therefore, extensive efforts have been made to produce these alkaloids through *in vitro* systems. On the other hand, in tropical areas where periwinkle can be easily cultivated, very little work has been done on increasing the yield of periwinkle alkaloids through improved agronomy, probably, because measurement of alkaloids in the plant material requires sophisticated and expensive instruments, technically trained personnel and is also time consuming.

More than 2600 patents have been granted on various inventions relating to use of this plant after the grant of first patent in 1961 (GB870723 19610621) on isolation of alkaloid Vincaleucoblastine from *Vinca roseus*. There has been a noticeable increase in number of patents granted from the year 2004, with the number of patents granted exceeding the number of publications suggesting increased commercial interest in periwinkle both as a medicinal and a horticultural plant (Supplementary Fig. 1S). Patents have been granted on a wide variety of inventions such as, use of its herb or its chemical constituents as medicine in various formulations or as drugs, including drug carrier, for novel methods of extraction or synthetic processes for its chemical constituents/alkaloids or and their derivatives, use of tissue culture and hairy root cultures for production of anti-cancer alkaloids, methods to improve flower colour, methods for enhancement of alkaloid production by using polyploid cells, methods for plant male sterility, enhancement of plant biomass, salt and low-temperature resistance, new plant varieties and so on.

Literature on biosynthesis of periwinkle alkaloids, their production *in vitro* through cell and tissue culture, and metabolic engineering is voluminous and has been periodically reviewed (van der Heijden *et al.*, 1989; Moreno *et al.*, 1995; Pasquali *et al.*, 2006; El-Sayed and Verpoorte, 2007; Za´rate and Verpoorte, 2007; Zhao and Verpoorte, 2007; Zhou *et al.*, 2009; Verma *et al.*, 2011; Moudi *et al.*, 2013; Salim and De Luca, 2013; Zhao *et al.*, 2013; Duge de Bernonville *et al.*, 2014; Matsuura *et al.*, 2014; Zhu *et al.*, 2014). The biosynthesis of terpenoid indole alkaloids (TIAs) in periwinkle is a multi-step complex process consisting of more than 50 biosynthetic events involving many genes, enzymes, regulators, intracellular transporters, organs, tissues and cell organelles and is regulated by ontogenic, environmental, organ- and cell-specific factors (Roepke *et al.*, 2010; Zhu *et al.*, 2014). It is highly compartmentalized with different portions of the pathways occurring in chloroplasts, the cytosol, the endoplasmic reticulum, the nucleus and vacuoles (Pasquali *et al.*, 2006;

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

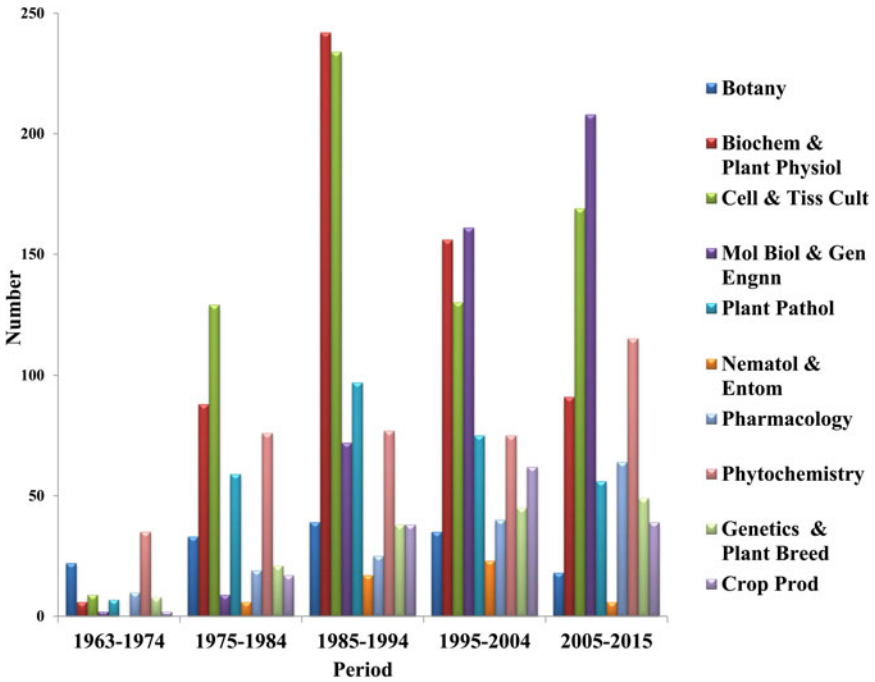

Fig. 2. Number of publications on periwinkle in different subject areas during the period 1963–2015.

Guirimand *et al.*, 2011). These TIAs are synthesized from secologanin (a monoterpenoid) and tryptamine (an indole) derived, respectively, from geranyl diphosphate *via* plastidial methyl erythritol phosphate pathway, and tryptophan *via* plastidial shikimate pathway. Tryptamine is synthesized from tryptophan by the enzyme tryptophan decarboxylase (TDC). The condensation of secologanin and tryptamine by the enzyme strictosidine synthase (STR) yields strictosidine, the central intermediate of all TIAs. Enzymatic deglucosylation of strictosidine through strictosidine-β-D-glucosidase (SGD) results in 4, 21-dehydrogeissoschizine, which is then catalysed by many enzymes in different branches to produce diverse TIAs. One of these branches leads to formation of cathenamine. Cathenamine is further converted into ajmalicine, serpentine, stemmadenine and the monomeric precursors (vindoline and catharanthine) of anti-cancer alkaloids, VLB and VCR, through different sub pathways (van der Heijden *et al.*, 2004; Pasquali *et al.*, 2006). At present, no genes, enzymes or intermediates involved in the catharanthine pathway have been characterized (Saiman, 2014) except for the identification of an unique catharanthine transporter gene (CrTPT2) that is expressed predominantly in the epidermis of young leaves and is responsible for accumulation of catharanthine entirely in the wax exudates on the leaf surface (Yu and De Luca, 2013). Biosynthesis of vindoline involves six enzymatic steps starting from the intermediate tabersonine (synthesized from strictosidine-aglycone), with last two steps of vindoline biosynthesis occurring in specialized cells, laticifer and idioblast cells, of aerial leaf mesophyll tissues (Salim and De Luca, 2013). Spatial separation of vindoline and catharanthine, the precursors of VLB and VCR, thus provides a clear explanation for the low levels of VLB and VCR in intact plants (Yu and De Luca, 2013). The coupling of vindoline and catharanthine to form anhydrovinblastine occurs in the vacuole through a peroxidase-like enzyme (Pasquali *et al.*, 2006).

Attempts to produce VLB and VCR through callus and cell culture have not been successful. Although catharanthine is produced in cell cultures, vindoline is not synthesized in undifferentiated cell and callus cultures. Vindoline biosynthesis requires shoot formation and the last biosynthetic step in vindoline biosynthesis occurs synchronously with shoot formation (Zhao *et al.*, 2001; Wink *et al.*, 2005; Pasquali *et al.*, 2006; Campos-Tamayo *et al.*, 2008; Salim and De Luca, 2013). Organ/shoot cultures are, however, difficult to grow in large-scale bioreactors and the yields of these anti-cancer alkaloids have been low and, therefore, do not provide an economically viable alternative to field-grown periwinkle plants as a source of these alkaloids (Wink *et al.*, 2005; Pasquali *et al.*, 2006; Roepke *et al.*, 2010).

Significant advances have been made recently in metabolic engineering of biosynthetic pathway of TIAs in heterologous organisms. The secologanin pathway is considered to be the rate-limiting step in TIA production in *C. roseus* cell cultures (van der Heijden *et al.*, 2004). Miettinen *et al.* (2014) discovered the last four missing

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

enzymes in the secologanin pathway and demonstrated heterologous production of strictosidine by reconstituting the entire TIA pathway up to strictosidine in *Nicotiana benthamiana*. Similarly, Brown *et al*. (2015) demonstrated production of strictosidine in *Saccharomyces cerevisiae* by engineering 14 known monoterpene indole alkaloid pathway genes. Since strictosidine is the central intermediate of all TIAs, its production in *S. cerevisiae* and *N. benthamiana* is a significant step in achieving heterologous production of periwinkle TIAs such as VLB and VCR. However, economic viability of further production of valuable periwinkle alkaloids, VLB and VCR, and or their precursors, vindoline and catharathine, *vis-à-vis* their extraction from field-grown plants still requires to be evaluated. At present, no economically viable alternative to field-grown plants is available for large-scale production of these alkaloids. Therefore, increasing yield of these alkaloids through appropriate crop management by identifying environmental and regulatory factors influencing alkaloid production in field-grown plants (Pasquali *et al*., 2006) and breeding periwinkle varieties with higher concentrations and yield of these periwinkle alkaloids (Sharma *et al*., 2012; Chaudhary *et al*., 2013) have been suggested. In this paper, published information on botany, cytology, genetics and breeding of periwinkle as a medicinal plant has been reviewed to gain an understanding of the constraints and possibilities of increasing the contents and yields of alkaloids in periwinkle at the plant level.

Botany

Origin and distribution

Periwinkle (*C. roseus*) is considered to be a native of the West Indies but was originally described from Madagascar (Ross, 1999). It was introduced into Paris in 1757 and has now become naturalized in continental Africa, America, Asia, Australia and Southern Europe and on some islands in the Pacific Ocean (van der Heijden *et al*., 2004). It is commonly grown as an ornamental plant throughout tropical and subtropical regions of the world by virtue of its wide adaptability, ever blooming nature and variously coloured flowers.

Taxonomy

The genus *Catharanthus* consists of eight species, seven of them viz., *C. roseus* (L.) G. Don, *C. ovalis* Markgraf, *C. trichophyllus* (Baker) Pichon, *C. longifolius* (Pichon) Pichon, *C. coriaceus* Markgraf, *C. lanceus* (Bojer ex A. DC.) Pichon, *C. scitulus* (Pichon) Pichon, indigenous to Madagascar and one viz., *C. pusillus* (Murray) G. Don, indigenous to India (Stearn, 1975).

Periwinkle (*C. roseus*) was designated variously earlier as *Vinca rosea* L., *Lochnera rosea* Rchb. and *Ammocallis rosea* Small. In 1753, Linnaeus established the genus *Vinca* and included two species *Vinca minor* and *Vinca major*. In 1759, he added tropical *V. rosea* to the temperate genus *Vinca* although it did not fit his generic description with regard to stamens. Reichenbach was the first to recognize *V. rosea* as generically different from *Vinca* (Stearn, 1975). In 1835, George Don while describing the genera retained the name *Vinca* for the genus containing *V. minor, V. major* and *Vinca herbaceae*, and gave the name *Catharanthus* to the genus typified by *V. rosea* and described it as *C. roseus* (Stearn, 1975).

The Taxonomic Hierarchy of *Catabaranthus* as described by Interagency Taxonomic Information System (ITIS) is given below (http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=30167#).

Kingdom: *Plantae* – plantes, Planta, Vegetal, plants
Subkingdom: *Viridiplantae*
Infrakingdom: *Streptophyta* – land plants
Superdivision: *Embryophyta*
Division: *Tracheophyta* – vascular plants, tracheophytes
Subdivision *Spermatophytina* – spermatophytes, seed plants, phanerogames
Class: Magnoliopsida
Superorder: *Asteranae*
Order: *Gentianales*
Family: *Apocynaceae* – dogbane, apocyns
Genus: *Catharanthus* G. Don – periwinkle
Species: *Catharanthus roseus* (L.) G. Don – Madagascar Periwinkle

Botanical description of the plant

Periwinkle is an annual or perennial semi-shrub growing to a height of 75 cm–1 m, sub-woody at the base and profusely branched. Stem colour – yellowish green, light pink or dark purple. Leaves, petioles and twigs contain milky latex. Leaves – oblong or ovate, opposite, short-petioled, smooth or pubescent with entire margin. Flowers – borne in pairs in axils, pedecellate, bracteate, hermaphrodite, actinomorphic, complete, hypogynous and pentamarous. Calyx – five parted, the sepals free almost to the base. Corolla – five lobed, small to large, salver-shaped, rose or white; tube cylindric, throat bearded, slender, externally swollen at the insertion of the stamens but contracted at the mouth; lobes free or overlapping, aestivation convolute. Stamens – five, attached to the middle of the corolla tube or just below the mouth, conniving over the stigma; filaments very short, not geniculate; anthers free from the stigma, dorsifixed, the connective not prolonged into an apical appendage, anther 2.5 mm long and the filament about 0.3 mm long, at anthesis. Carpels – two, distinct,

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

narrowly triangular glands present at the base of the carpels; ovules, numerous (about 10–30) in two series in each carpel; style long, slender; clavuncle shortly cylindric, truncate at base. Carpels united only by the style at the apex. Stigma capitate, bearded at the top and furnished with a cup-shaped/'skirt' like membrane below, which sheaths the upper part of the style. Fruit consists of two long cylindric pointed follicles (mericarps) diverging or parallel, containing 10–30 seeds, dehiscent at maturity along the length. Seeds – numerous, small (1.5–3.0 mm long), oblong, cylindrical, not arillate, with the hilum in a longitudinal depression on one side, blackish, muriculate, the surface minutely reticulate.

Reproductive biology

Flower development

A knowledge of the morphology of the flower, its development, anthesis, pollination mechanism, mode of pollination, presence or absence of incompatibility, fruit and seed set of the plant is an essential prerequisite for understanding the breeding behaviour of the plant species. Information on these aspects is necessary for developing artificial selfing and hybridization techniques, maintenance of varietal purity and for choosing and executing appropriate breeding methodology.

Flowering in periwinkle begins when plants are about 10–15 cm tall or about 10 weeks old and continue to flower as long as plants live. Flowers appear in pairs in the alternate leaf axils and the two flowers are never in the same stage of development, with one flower opening approximately 2–3 d before the other.

The vegetative phase is very short and the adult shoot apex is associated with flowering. After many meristematic divisions in the shoot apex, sepals are initiated. Following sepal initiation, the floral apex enlarges rapidly and becomes broad and flat. Petals arise at the edge of the floral meristem, adaxial to and alternate with the sepals (Boke, 1948). In early stages of development, the flower is polypetalous. The corolla tube begins to form shortly after carpel initiation. The upper corolla tube (part above insertion of stamens and below corolla lobes) is formed by ontogenetic union of bases of petals. The lower corolla tube (i.e. below insertion of stamens) is formed by intercalary growth in the common bases of petals and stamens. The region below the stamen continues to elongate until the flower opens (Boke, 1948).

Stamen primordia appear soon after petal initiation. After a sequence of cell divisions, the stamen at first appears to consist of sessile anthers and later the filaments are formed. Following the stamen initiation, the floral apex flattens. The central region grows at a slow rate and later develops into carpel. The carpels are sessile and are not united with each other in the ovary region. The style remains short until the flower is nearly mature (Boke, 1948, 1949; Rendle, 1971).

Anthesis and pollination

The corolla tube grows faster than the limb up to the day of flowering (Simmonds, 1960). The elongation of style corresponds with the elongation of the corolla tube, so that the relative positions of the stigma and stamens remain almost constant. As the flower approaches anthesis, epidermis of the capitate stigma forms secretory cells on the sides and a curious 'skirt' (a cup-shaped membrane) at its base. Based on these observations on the development of stamens and carpels, Boke (1949) concluded that the flower is well adapted for self-pollination.

Under South Indian conditions, anthesis generally starts from 15.00 to 16.00 h and continues until the next day. Anther dehiscence occurs just before anthesis and the pollen is shed as a sticky mass (Supplementary Fig. 2S). The stigma was found to be receptive between 06.00 and 10.00 h, and between 15.00 and 17.00 h (Sreevalli, 2002).

Sprengel (Knuth, 1909) and Boke (1949) supposed that periwinkle flower was adapted for self-pollination. Periwinkle has also been considered as a self-pollinating species in several other studies also (Flory, 1944; Simmonds, 1960; Krishnan *et al.*, 1979; Levy *et al.*, 1983; Milo *et al.*, 1985). As periwinkle plants were generally found to be true breeding for their flower colour, Flory (1944) and Simmonds (1960) considered that periwinkle is a self-pollinating species. Krishnan *et al.* (1979) used pink flower colour as the marker trait for determining out-crossing and found that natural out-crossing in periwinkle ranged from 1.7 to 13.8% in white flowered plants. They opined that periwinkle is essentially a self-pollinating plant with not infrequent out-crossing, as flowers remain open up to 3 d for possible insect pollination.

A characteristic feature of *Apocynaceae*, is the disc-like or otherwise-shaped enlargement of the stigma-head with a sticky secretion and a brush of hairs on which pollen collects as it is shed. The receptive portion of the stigma is at the base of the stigma head, and owing to the position of the anthers, self-pollination is rendered almost impossible, and insect-visits are necessitated (Rendle, 1971). Pollination is brought about by nectar-seeking insects, which effect pollination by depositing pollen collected from the flowers during their previous visits (Knuth, 1909). Automatic self-pollination in periwinkle is thus excluded. Experimental studies have also shown that automatic self-pollination does not occur in periwinkle and pollinators are necessary to bring about pollination (Kulkarni, 1999; Sreevalli *et al.*, 2000). Further, two pollinating butterflies (Supplementary Fig. 3S), *Pachliopta hector* and *Catopsilia pyranthae*, have been found to exhibit flower colour constancy during their flower visits and cause

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

about phenotypic assortative mating for flower colour resulting in greater number of intra-flower colour matings than inter-flower colour matings. This phenotypic assortative mating for flower colour combined with geitonogamy could result in plants appearing to be breeding true for their flower colour on open-pollination (Kulkarni, 1999). Many pollinators *Bombylius discolor, B. major, Anthophora pilipes, Apis mellifica, Bombus agrorum, B. hortorum, B. hypnorum, B. pratorum, B. terrester, B. vestalis, Osmia fusca, O. rufa* and thrips have been observed in the genus *Vinca* (Knuth, 1909).

Some self-pollinating strains have, however, also been found in periwinkle. In these strains, self-pollination occurs after anthesis due to continuous elongation of either ovaries or styles until pollination by overcoming spatial separation of stigma and stamen. Thus, both types of pollination occur in the periwinkle (Kulkarni *et al.*, 2001, 2005a).

Fertilization and embryology

The mature megagametophyte is normal or polygonum type with ephemeral type of antipodal cells. The microsporangium contains 92–96% viable pollens. The pollen tube reaches the megagametophyte within 12–24 h after pollination. Fertilization is porogamous type and usually occurs 48–72 h after pollination. Endosperm is of nuclear type and endosperm wall formation starts from the periphery towards the centre. The embryo development is caryophyllad type (Dnyansagar and Sudhakaran, 1977).

The corolla remains persistent generally for 2–3 d but generally falls off before fruit set becomes visible. Fruit (follicle) matures in 4 to 5 weeks. The follicles are dehiscent and shed seeds as they dehisce by the ventral suture. Seeds generally exhibit a period of dormancy of about 3–4 weeks and have been found to remain viable for about 12–18 months at room temperature under tropical – subtropical conditions.

Artificial hybridization and selfing techniques

Artificial hybridization and selfing techniques have been employed for carrying out genetic studies. Flower buds have been emasculated 1 d before anthesis (Kulkarni *et al.*, 2001) or 2–3 d before anthesis (Levy *et al.*, 1983; Sevestre-Rigouzzo *et al.*, 1993) by making a partial cut, about 1 mm, above the base of the throat of the corolla tube. The top portion of the flower bud was then removed along with the undehisced anthers. (Kulkarni *et al.*, 2001). Pollination of emasculated flower buds was carried out on the same day of emasculation (Kulkarni *et al.*, 2001) or 2–3 d after emasculation (Levy *et al.*, 1983) or twice a day for 3 d after emasculation (Sevestre-Rigouzzo *et al.*, 1993). Pollinated flower buds were protected by paper bags or pieces of plastic straw (closed at the top end) to prevent accidental pollination and seed loss at the maturation stage. The per cent fruit set ranged from 90 to 100% (Sevestre-Rigouzzo *et al.*, 1993; Kulkarni, 1999).

Cytology

Chromosome number

Periwinkle is a diploid plant species with a chromosome number of $2n = 16$ (Janaki Ammal and Bezbaruah, 1963; Dnyansagar and Sudhakaran, 1968, 1970; Stearn, 1975; De Padua *et al.*, 1992). Out of eight pairs of chromosomes, four pairs were found to have submedian centromeres and two each subterminal and median centromeres (De Padua *et al.*, 1992; Jia *et al.*, 2008; Guimarães *et al.*, 2012). Few abnormal cells with $2n = 12$ and 14 chromosomes were also observed (De Padua *et al.*, 1992). The average length of chromosomes ranged from 4.784 to 8.627 µm (Guimarães *et al.*, 2012).

Based on karyomorphological studies of *C. pusillus* and six varieties of *C. roseus*, Rani and Kumar (2011) concluded that *C. pusillus* was evolutionarily more primitive and white-flowered variety of *C. roseus* was most advanced. They further observed secondary constrictions on short arm of three out of eight pairs of chromosomes of *C. pusillus* but none in *C. roseus*. However, Guimarães *et al.* (2012) observed the presence of the nucleolar organizer region in chromosome 6.

Dnyansagar and Sudhakaran (1968) studied chiasma frequency, type of bivalents at diakinesis and metaphase I of meiosis in pink and white varieties of periwinkle. The percentage of ring bivalents was found to be 73% at diakinesis and 72% at metaphase I. The chiasmata per bivalent varied from 2.8 to 1.7% from late diplotene to metaphase I. The mean number of chiasmata per cell at the early metaphase I was 13.7 and did not vary between varieties.

Genome size

The nuclear DNA content (1C) of periwinkle has been estimated to be 0.70 and 0.76 pg corresponding to 696 and 738 Mbp by Zonneveld *et al.* (2005) and Guimarães *et al.* (2012), respectively. The quantity of DNA and molecular size of chromosomes of *C. roseus* ranged from 0.070 to 0.127 pg and 69 to 124 Mbp, respectively (Guimarães *et al.*, 2012).

The complete plastome of *C. roseus* (154,950 bp in length) has been sequenced and 41 *C. roseus*-specific plastome (SSR) markers with potential utility for *C. roseus* breeding and phylogenetic analyses have been identified. Complete plastome sequence could be used to engineer the *C. roseus* plastome to accelerate synthesis of

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

isopentenyl pyrophosphate (IPP), the rate limiting step for alkaloid accumulation which occurs in plastids (Ku *et al.*, 2013).

Genetics

Genetics of corolla colour

Flower or corolla colour is the most conspicuous trait for which variation is easily apparent in periwinkle populations. Periwinkle owes its horticultural importance to its variously coloured flowers. Flower colour was, therefore, the first trait whose inheritance was studied. In natural populations, mainly three basic corolla colour patterns are observed viz., pink, red-eyed (white corolla with red centre) and white. Flory (1944) attributed epistatic interaction between two genes *R* and *W* to explain flower colour differences in these three phenotypes. In a cross between pink and white flowers, he observed a ratio of nine pink: three red-eyed: four white-flowered plants; with genotypes *R–W* being pink, *R-ww* red-eyed and *rrW-* and *rrww* being white flowered. Red pigmentation in stems of pink-flowered plant was associated with flower colour. Thus, stem colour indicated flower colour prior to flowering. According to Simmonds (1960), two more genes (*A* and *B*) are involved in the determination of flower colour. Gene *A* is a basic colour gene, which is complementary to gene *R* of Flory (1944), without both of which the flower colour is white. Gene *B* is a co-pigmentation gene, which blues the pigment in pink- and red-eyed flowers resulting in violet- and purple-eyed flowers, respectively. In addition to the above three corolla colours, Milo *et al.* (1985) identified another flower colour namely, pale pink centre and attributed this corolla colour to another gene *I* which is also epistatic to gene *R* like the gene *W*. The gene *I* produces pigment in the centre of the corolla but in smaller amounts than gene *W*.

Mode of inheritance of other corolla colours viz., orange-red corolla and magenta corolla and the presence or absence of red eye has also been studied (Sreevalli *et al.,* 2002; Kulkarni *et al.*, 2005b, 2008). Epistatic interactions between independently inherited genes *R, W, B, I, O,* O^m*, J* and *E* were found to be responsible for the production of pink, white, violet, pale pink, orange-red, magenta, scarlet-red and rose corolla with or without red eye (Supplementary Fig. 4S). Gene *E* determines the presence or absence of red eye, i.e. *R* allele produces pigment in the eye region only in the presence of allele *E*; in its absence flowers have white eye. Allelic genes *O* and O^m produce orange-red and magenta corolla, respectively, only in the absence of *W* allele. Heterozygotes with OO^m alleles at *O* locus produce scarlet-red corolla. Rose corolla is produced by inhibitory interaction between O^m and *J* alleles.

A large number of cultivars with a range of corolla colours have been developed by horticulturists (Snoeijer, 2001).

Genetics of resistance to die-back disease

Die-back disease, caused by *Pythium aphanidermatum*, is a devastating disease in the rainy season in the tropical and subtropical regions. High mortalities up to 70–80% have been reported (Pareek *et al.*, 1981; Kulkarni, 1984). The pathogen also causes collar and root rot resulting in the death of plants. A die-back resistant variety named 'Nirmal' was developed as a pure line selection from a single plant that survived in a severe die-back epidemic (Kulkarni *et al.*, 1999). Inheritance of resistance to die-back was studied using a die-back resistant dwarf mutant of variety, Nirmal, with a green stem, and a susceptible accession, 'OR', with a purple stem. From both quantitative and qualitative analyses of data, resistance to die-back appeared to be governed by a single gene (with a broad-sense heritability of 0.85) and was inherited independently of genes governing dwarfness and stem pigmentation. Resistance of variety, Nirmal to dieback has remained durable for the last nearly 30 years (Kulkarni and Baskaran, 2003).

Genetics of mechanisms of pollination

The structure of periwinkle flower is of typical reverse herkogamy where the stigma is below the anthers and automatic intra-flower self-pollination is excluded. Pollination occurs through nectar-seeking insects. However, self-pollinating strains have also been found. Studies on the mechanism of self-pollination in these strains revealed two different mechanisms, one in which self-pollination is brought about by the continuous elongation of the style, and the other by the continuous elongation of the ovary till pollination (Supplementary Fig. 5S) The two mechanisms were found to be governed by duplicate alleles recessive to allogamy ($SP_1SP_1SP_2SP_2$), with alleles ($sp^o{}_1sp^o{}_1$-$sp^o{}_2sp^o{}_2$) governing ovary elongation being dominant to alleles ($sp^s{}_1sp^s{}_1sp^s{}_2sp^s{}_2$) governing style elongation (Kulkarni *et al.*, 2005a). However, self-pollination in these strains occurred 1–2 d after anthesis and thus, out-crossing is not ruled out under conditions of open pollination. Nevertheless, genes governing self-pollination can be transferred to desirable genotypes and their genetic purity maintained through seeds produced by autonomous self-pollination in the absence of pollinators or under isolation.

Development of cleistogamy

Self-pollination, brought about by increase in the length of gynoecium (ovary or style) in self-pollinating strains

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

described above, was found to occur after 1–2 d after anthesis. Therefore, maintenance of genetic purity is not automatically ensured in these self-pollinating strains. Cleistogamy, if developed in periwinkle, would facilitate maintenance of genetic purity without manual selfing and seed production without dependence on pollinators. It would also ensure pollen containment which is an important requirement in the development of transgenics. An ethyl methanesulphonate (EMS)-induced mutant, in which corolla abscised before opening of the corolla, i.e. at the flower bud stage was identified. By crossing this mutant with self-pollinating strains described in the previous section, cleistogamous strains were developed (Supplementary Fig. 6S). These strains constitute pyramiding of recessive alleles at four loci, closed corolla, normal plant height (or a tightly linked gene inhibiting closed corolla) and self-pollination (Kulkarni and Baskaran, 2013a).

Breeding

Assessment of genetic variability and genetic divergence

Information on the breeding system of the plant, germplasm resources available, extent of genetic variability for traits of interest, their heritabilities, inter-trait correlations and genetic divergence in the germplasm is the basic prerequisite for initiating breeding work in any plant species of economic interest.

Although periwinkle attained prominence as a source of anti-cancer alkaloids in the late 1950s, studies on genetics and breeding aspects of this plant were few and sporadic till the late 1990s. In perhaps, the first study, Levy *et al.* (1983) reported marked differences for yields of leaves and roots and for contents of ajmalicine in roots of three unrelated pure lines representing three flower colour types: pink corolla, white corolla and white corolla with red eye. The differences between lines varied according to developmental stage of the plant. They also observed 29 and 24% significant and positive heterosis over better parent for leaf and root dry yields per plant, respectively, in the F_1 hybrid involving the parental lines, pink corolla, and white corolla with red eye. However, no heterosis was observed for ajmalicine content in roots. They suggested breeding of pure line cultivars with high yields of leaves and roots and high contents of alkaloids in leaves and roots till the availability of male sterile lines for exploiting the observed heterosis for the leaf and root yield. The absence of heterosis for ajmalicine content in roots did not favour the development of hybrid cultivars of periwinkle for use by the pharmaceutical industry.

Virk *et al.* (1988) identified two genotypes with high yields of leaf and root alkaloids for direct cultivation after 2-year evaluation of 17 genotypes collected from diverse sources such as, Poland, the then USSR and different parts of India. Significant genotype × year interaction was also found for a majority of the traits studied by them, implying that ranking of genotypes was dependent on the year of evaluation. Genetic variability, heritability of different traits, genetic advance and genetic diversity among selected 20 M_8 lines and six other selected lines were determined by Dwivedi *et al.* (1999, 2000). Wide variation was observed for plant height, number of branches per plant, leaf yield, and for the contents of total alkaloids, vindoline, catharanthine, VCR and VLB. High broad-sense heritabilities (83–91%) were observed for leaf yield and for the contents total alkaloids, vindoline, catharanthine, VCR and VLB suggesting that the differences between the studied genotypes were mainly due to genetic causes. However, recently Sharma *et al.* (2012) found moderate to moderately high heritabilities for leaf yield, root yield and for contents of vindoline, VLB, catharanthine in leaves (40–79%) and low heritabilities for contents of total alkaloids in leaves and roots (5 and 13%). The 26 entries evaluated by Dwivedi *et al.* (2000) fell in to nine clusters, I–IX. Wide diversity was observed between clusters VIII and IX, and clusters VI and VIII, suggesting hybridization between entries belonging to these clusters for realizing greater magnitude of heterosis and wide genetic variability in the segregating generations.

In a relatively larger study, Mishra *et al.* (2001) evaluated 32 accessions collected from wide geographical areas such as different regions of the Indian sub-continent, Sri Lanka, Madagascar, Singapore and Malaysia for 53 growth, development and alkaloid yield-related characters over two seasons. Large differences were observed between accessions for six morphological and 14 agronomic traits; the differences were 3, 80 and 15-fold for the main alkaloid yield components viz., leaf dry matter yield, VLB and VCR contents, respectively. The 32 accessions fell into seven clusters by principal component analysis. Five accessions from tropical areas formed separate cluster (cluster 1). The largest cluster (cluster 2) had 16 accessions from semi-tropical to semi-temperate geographical areas. However, four accessions also from semi-tropical to semi-temperate geographical areas separated into cluster 3. Four accessions from heavy rainfall and humid areas formed cluster 5. One accession each from Andaman Islands and Singapore, and a mutant cultivar separated from each other as well as from rest of the accessions. Hierarchical UPGMA analysis grouped the accessions into five clusters. In general, the genetic base of periwinkle population from the Indian subcontinent and surrounding Asian region appeared to be narrow as half of the accessions from wide geographical distances were placed in the same clusters by multivariate and hierarchical UPGMA analyses.

Diversity among 32 *C. roseus* accessions obtained from different geographical regions of India, Sri Lanka,

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

Mozambique and private seed companies from India and Sweden, and one accession each of *C. trichophyllus, C. pusillus, Vinca minor, Thevetia peruviana* and *Nerium indicum* was assessed for the first time using sequence-tagged microsatellite site (STMS) markers (Shokeen *et al.*, 2007). Non-*Catharathus* species separated clearly from *Catharathus* cluster. *Catharathus trichophyllus, C. pusillus* separated from other *C. roseus* accessions within *Catharathus* group. Within the *Catharathus* group, the accessions were generally grouped according to their geographical origin (Shokeen *et al.*, 2007)

Inter-trait correlations

Information on inter-trait correlations is essential to know the effect of selection for one trait of interest on other unselected traits, and to know the possibility of carrying out indirect selection for characters of interest which are difficult or time consuming to measure, or are less heritable. In periwinkle, estimation of contents of total alkaloids and specific alkaloids viz., VLB, VCR, vindoline, catharanthine and ajmalicine is time consuming and limits the number plants that can be evaluated in a breeding programme. Any trait with high heritability and a strong correlation with contents of these alkaloids could be useful for preliminary screening for content of alkaloids as well as for indirect selection for these important traits.

Leaf yield and root yield, leaf yield and leaf alkaloid yield, root yield and root alkaloid yield were found to be positively correlated suggesting that simultaneous improvement for these pairs of traits should be possible through selection (Mishra *et al.*, 2001; Sharma *et al.*, 2012). Leaf yield and root yield were not correlated with leaf alkaloid concentration and root alkaloid concentration, respectively (Mishra *et al.*, 2001). Therefore, it should be possible to combine high yield of these two plant parts with high concentrations of alkaloids in them. The content of total alkaloids in leaves was positively correlated with contents of catharanthine, vindoline and VLB in leaves (Sharma *et al.*, 2012) suggesting that selection for total alkaloids in leaves should be effective in improving contents of catharanthine, vindoline and VLB in leaves. As expected, the contents of catharanthine, vindoline and VLB in leaves were positively correlated (Sharma *et al.*, 2012; Chaudhary *et al.*, 2013).

No relationship was found between flower colour and contents of vindoline and catharanthine in 50 horticultural cultivars which had been bred for flower colour. However, one of the cultivars had low content of vidoline and ten times lower tabersonine-16-hydroxylase activity as compared with *C. roseus* cv. Little Delicata (Magnotta *et al.*, 2006).

Interspecific hybridization and exploiting wild species

Although the genus *Catharanthus* consists of eight species (including *C. roseus*), no attempt has been made to evaluate and exploit interspecific variability. *Catharanthus roseus* is considered to be incompatible with other *Catharanthus* species. However, natural hybridization between periwinkle species has been observed in Madagascar and most of these hybridizations were between *C. longifolius* and *C. roseus*. Reciprocal differences were observed in crossability between *C. roseus* and *C. trichophyllus*. *Catharanthus roseus* as female parent failed to form fruits and therefore, no introgressions were found from *C. trichophyllus* to *C. roseus* (Sevestre-Rigouzzo *et al.,* 1993). In reciprocal cross, however, up to 100% seed set with good germinability was found. Alkaloid profiles of *C. trichophyllus* and *C. roseus* differed with absence of serpentine in leaves of *C. trichophyllus* and catharanthine in roots of *C. roseus*. Hybrids contained serpentine in leaves such as leaves of *C. roseus* and catharanthine in roots such as roots of *C. trichophyllus*. Further, significant heterosis was found for the contents of ajmalicine, catharanthine and serpentine both in leaves and roots and for the content of vindoline in leaves. The hybrids also had higher leaf and root yields than the parental species. Therefore, they suggested development of hybrids coupled with micro-propagation to exploit observed heterosis for alkaloid production.

Induced autotetraploidy

Induced autopolyploids generally have larger and thicker leaves, stems, roots, flowers and fruits. Autopolyploidy has also generally been found to enhance production of secondary metabolites (Dhawan and Lavania, 1996; Lavania, 2005; Lin *et al.*, 2011; Dehghan *et al.*, 2012; Lavania *et al.*, 2012; Madani *et al.*, 2015). In plant species in which vegetative parts are economically important and where very little genetic improvement work has been done, induced autopolyploidy could be a rapid method of increasing the yield of their vegetative parts. Further, plant species which have lower chromosome number are considered to be better suited for development of autotetraploids superior to their diploids than those which have higher chromosome number. It is likely to be less successful in plant species where seed is the commercial product and is the only method of propagation. Periwinkle has a low chromosome number and its vegetative parts, leaves and roots, and not seeds, are of economic importance. Therefore, many attempts have been made to induce and evaluate autotetraploids.

There are numerous reports on induced autotetraploidy in periwinkle (Janaki Ammal and Bezbaruah, 1963;

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

Dnyansagar and Sudhakaran, 1970, 1977; Mohan Kumar, 1980; Kulkarni *et al*., 1984, 1987; Krishnan *et al*., 1985). To induce tetraploidy, either seeds or apical buds of young seedlings were treated with different concentrations of colchicine solutions ranging from 0.01 to 1.0%. Colchicine treatment of apical buds was more effective in inducing tetraploidy than seed treatment. Increase in the length of stomata and pollen grain diameter have been observed as the two characteristic effects tetraploidy in periwinkle also.

Autotetraploids were found to be more vigorous in growth with broader leaves, larger stomata, flowers, pollen grains and embryos but had low pollen fertility, poor fruit set and low seed production as compared with diploids (Janaki Ammal and Bezbaruah, 1963; Mohan Kumar, 1980). In autotetraploids, pollen fertility was low (32–43%) leading to poor seed set (17.5–22.5%). Prefertilization abnormalities such as undeveloped ovules, lack of normal organization or delayed organization of the embryosac and delayed fertilization were responsible for the breakdown of seed formation in the autotetraploids (Dnyansagar and Sudhakaran, 1977). Polyploidization did not affect the normal pattern of development of embryo but increased the rate of growth of embryo and endosperm. Embryo grew faster during the early period, while in later stages the growth seemed to slow down, whereas endosperm development was slow initially and faster later. Tetraploid seeds took longer time (28–32 d) to mature than the diploid seeds (24 d). Seed development in autotetraploids was similar to that in diploids; however, polyploidization altered the growth of embryo and endosperm therein. The embryo size was larger in tertaploids than in diploids (Dnyansagar and Sudhakaran, 1977).

No consistent effects of autotetraploidy have been found on leaf yield, root yield and content of total alkaloids in leaves and roots in periwinkle. In some studies (Dnyansagar and Sudhakaran, 1970; Mohan Kumar, 1980), autotetraploids had significantly higher leaf yield, root yield and content of total alkaloids than diploids, while in other studies (Kulkarni *et al*., 1984; Krishnan *et al*., 1985) they were found to be on par with diploids for leaf yield and root yield but had lower ajmalicine content and harvest index. Goswami *et al*. (1996), however, observed that tetraploids, generally, had lower leaf yields but some of them had higher contents of catharanthine, vindoline and VCR than their diploid parents. The observed differential effects of induced autotetraploidy on different traits may have been due to differences in the genetic makeup of diploid parental genotypes used.

Tetraploids performed better than diploids at close plant spacings (30×30 cm^2), especially in the absence of nitrogen application suggesting that tetraploids could be grown on soils with low fertility as a rain-fed crop for obtaining higher yields than diploids (Kulkarni *et al*., 1987). Further studies showed that tetraploids had higher nitrogen utilization efficiency than diploids (Kulkarni *et al*., 1995). Induced autotetraploids were also found to be highly resistant to die-back, and collar and root rot (devastating diseases in rainy season) and yielded four and five times more leaf and root total alkaloids, respectively, than diploids. Their resistance was equivalent to the protection provided by 5.76 kg/ha of a fungicide Captafol or soil solarization for a susceptible variety (Kulkarni and Ravindra, 1988, 1997; Kulkarni *et al*., 1992).

In a recent study, the contents of vindoline, catharanthine and VLB were found to be higher in tetraploid lines than in diploids and corresponded with higher levels of expression of the genes of *tdc, g10h, sls, str, dat* and *prx1* in the tetraploid lines as compared with the diploids (Xing *et al*., 2011).

Mutation breeding

Mutation breeding is generally adopted to create novel variation when genetic variation for desirable traits is not available in the germplasm or is available in undesirable genetic backgrounds. It is a powerful and effective tool in the hands of plant breeders for improvement of crops having narrow genetic base (Micke, 1988). The role of mutation breeding in increasing the genetic variability for desired traits in various crop plants has been proved beyond doubt. It is also perhaps the most rapid method of variety development. As per International Atomic Energy Association (IAEA) mutant variety database, 3220 mutant varieties have been released in 170 different plant species in more than 60 countries (Pathirana, 2011; Mba, 2013) and of these, about 2700 have been developed directly from induced mutants (Pathirana, 2011; Mba, 2013; Roychowdhury and Tah, 2013).

In industrial crops, such as medicinal plants, the content of the economically important metabolite is more important than the yield of the plant parts containing the metabolite because it determines the cost of extraction of the metabolite. Mutation breeding is one of the most promising approaches for the development of 'ideochemovars' (Swaminathan, 1972; Levy, 1982).

Mutation breeding has been adopted more frequently in self-pollinating crops than in cross-pollinating ones, due to failure of recessive mutations to express in cross fertilizing systems without manual selfing or sib-mating. Periwinkle, although a herkogamous species, was earlier considered to be a self-pollinating species because of geitonogamy and the need for artificial selfing was not realized. Nevertheless, periwinkle has been subjected to induced mutagenesis and several mutants affecting different traits including contents of alkaloids, with direct or indirect utility through hybridization, have been isolated. Estimation of contents of alkaloids is time consuming. So far no rapid

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

methods for estimation of contents of alkaloids are available for use in breeding programme. Although radioimmunoassays were developed long ago (Arens *et al.*, 1978) for rapid, accurate and reliable quantitative estimation of periwinkle alkaloids, to the best of our knowledge, we are not aware of their use subsequently in breeding programmes. In the absence of rapid methods for screening plants for their alkaloid contents, macro-mutants with altered morphology have been evaluated for identifying mutants for altered alkaloid contents. Induced macro-mutants in periwinkle include those with altered plant height, leaf morphology, floral traits, reproductive traits, and those with tolerance to salt, heat and water stress.

Mutants with altered plant height

Plant height mutants are one of the most commonly observed groups of mutants in induced mutagenesis programmes in different crop plants. Plant height is an important trait which along with shoot branching and inflorescence morphology determines plant architecture and crop yield (Wang and Li, 2006). Among plant height mutants, those with reduced plant height have generally been observed more frequently than those with increased plant height and have also been studied with greater interest due to their resistance to lodging and response to fertilizers. In fact, the 'Green Revolution' is attributed to the discovery of dwarfing genes 'Norin 10' and 'Dee-Gee-Woo-Gen' in wheat and rice, respectively.

Three distinct reduced plant height mutants, 'dwarf', 'semi-dwarf' and 'bushy', respectively, about 60, 40 and 30% shorter than their parental variety, Nirmal have been reported in periwinkle (Kulkarni *et al.,* 1999, 2009). The 'dwarf' and 'semi-dwarf' mutants were due to monogenic recessive genes (dw_1 and dw_2, respectively) which were allelic to each other and had significantly higher content of root alkaloids than parental variety. The 'bushy' mutant which was governed by an independently inherited non-allelic recessive gene (*by*), however, had similar contents of leaf as well as root alkaloids as the parental variety, Nirmal. The double-mutant recombinant ($bydw_1$) was 30% shorter than the shorter of the parental mutants and exhibited 20% higher content of root alkaloids than the better parent. All the three mutants and the double-mutant recombinant ($bydw_1$) had similar contents of leaf alkaloids. Higher root alkaloids content has been found to be related to thin root morphology in hairy root cultures of periwinkle (Palazon *et al.*, 1998). An extremely tall mutant, about 90% taller than the parental variety, Nirmal and controlled by epistatic inhibitory interaction between two independently inherited dominant genes has also been reported. The mutant, however, had similar leaf and root yields as well as contents of leaf and root alkaloids as the parental variety (Kulkarni and Baskaran, 2013b).

Mutants with altered leaf morphology

As economically important alkaloids are present in the leaves, altered leaf morphology may suggest altered alkaloid contents. Three leaf mutants, *viz*. wavy leaf margin, 'necrotic leaf' (a lesion mimic mutant) and 'nerium leaf' (resembling leaf lamina of another Apocynaceous plant, *Nerium oleander*) exhibited higher contents of leaf alkaloids than their respective parents (Kulkarni *et al.*, 1999; Baskaran *et al.*, 2013). Further, enhanced contents of leaf alkaloids of 'necrotic leaf' and 'nerium leaf' mutants over their parental variety were found to be due to recessive alleles at different loci, and 13 out 14 double-mutant recombinants for parental mutant traits 'necrotic leaf' and 'nerium leaf' developed by crossing the two mutants had significantly higher content of leaf alkaloids than parental mutants (Kulkarni and Baskaran, 2014).

No studies have been carried out on linkage between leaf alkaloids content and these morphological mutant traits. However, it appears that 'necrotic leaf' trait could be used as a seedling marker trait for enhanced content of leaf alkaloids. Extract of *Pythium* (a soil borne pathogen of periwinkle) is well known to be an elicitor of alkaloid production in cell and tissue cultures of *C. roseus* (Nef *et al.*, 1991). Therefore, constitutive expression of self-defence reactions in the 'necrotic leaf' mutant may have induced enhanced production of alkaloids similar to that elicited by *Pythium*. Transgenic tobacco (*Nicotiana tobacum*) plants expressing TDC exhibited necrotic lesions on leaves due to accumulation of high levels of tryptamine in the chloroplasts, which is poisonous to chloroplasts (Di Fiore *et al.*, 2002). Many induced lesion-mimic mutants with enhanced resistance to pathogens have been obtained in crop plants using different mutagens. Wu *et al.* (2008) obtained 21 independently induced lesion-mimic mutants using gamma rays, fast neutrons and diepoxybutane. Three of them were dominant, 17 recessive and one was dominant and maternally inherited. Two of these mutants showed enhanced resistance to multiple strains of rice blast (*Magnaorthe oryzae*) and bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) pathogens. Further, double-mutant recombinant of two independent lesion-mimic mutants showed higher level of resistance to bacterial blight pathogen than parental mutants indicating synergistic effect of parental mutations. The frequency of lesion-mimic mutants appears to be high in plants and more than 200 lesion-mimic genes have been estimated in maize alone (Johal *et al.*, 1995). Since these lesion-mimic mutants can be easily identified at seedling stage, screening populations for lesion-mimic mutants following treatment with different mutagens could be one simple way of identifying mutants with enhanced contents of alkaloids. It should be possible to further enhance the contents of alkaloids by developing double-mutant recombinants. However, not all lesion-

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

mimic mutants have been found to exhibit enhanced resistance to diseases and, also, not all leaf mutants in periwinkle have been found to have altered contents of alkaloids.

Mutants with altered floral and reproductive traits

Significant heterosis for leaf and root yields was found in one of the earliest genetic studies in periwinkle (Levy *et al*., 1983). However, for commercial exploitation of heterosis, male sterility is required for large-scale production of hybrid seeds. A patent has been granted for the method for developing hybrids in *Cathranthus* using male sterility (Bowman, 2000). Streptomycin was used to develop genetic male sterile line '13861–1' with msGS gene, which does not set selfed seed. The gene did not show any undesirable pleiotropic effect.

Two other types of male sterile mutants viz., indehiscent anthers (functional male sterility) and pollen-less anthers governed by a single recessive allele and duplicate recessive alleles, respectively, have also been reported (Sreevalli *et al*., 2003; Kulkarni and Baskaran, 2008). The mutant with indehiscent anthers had relatively smaller anthers and about 30% lesser number of pollen grains but was otherwise phenotypically normal, had high pollen fertility and showed high seed set on artificial selfing. The possibility using this mutant for hybrid seed production was studied by determining fruit- and seed-set in a small seed production plots planted with one row of the mutant surrounded by two rows of its parental variety. Good fruit-set and number of seeds per follicle were observed in the mutant line used as female parent. All the seedlings raised from a sample of hybrid seeds produced were found to be true hybrids. Since the mutant carried recessive genes for three early expressing traits, use of a dominant allele at one or more of these loci in the male parent was suggested to facilitate easy identification of true hybrids at seedling stage for transplantation in the field (Sreevalli *et al*., 2003). The mutant could be easily multiplied by manual selfing, vegetatively through stem cuttings or micro-propagation. The utility of the mutant in hybrid seed production would, however, be determined by economics of its multiplication *vis-à-vis* heterotic advantage of the hybrid. In contrast to these two male sterile mutants, another partially sterile mutant with *in situ* pollen germination has also been reported (Mishra and Kumar, 2001).

Mutations affecting the gynoecium have also been reported. In periwinkle flower, the stigma is about 0.5 mm below the anthers, typical of reverse herkogamy. Mutants with short style (about one-third length of normal style) and long style with stigma 2.5 mm above the tip of the cone of anthers, with partial and high pollen sterility, respectively, have been reported (Mishra and Kumar, 2003; Kulkarni and Baskaran, 2008). The short-styled mutant trait was inherited as a recessive trait, while the long-styled mutant constituting 'pin' flower in contrast to 'thrum' flower in normal plants appeared to be under the control of inhibitory, epistatic interaction between two independently inherited genes *P* and *T*, with gene *T* being inhibitory to gene *P*. Accordingly, genotypes *P-T-, T-pp*, or *pptt* produce normal 'thrum' flowers whereas *P-tt* produce mutant 'pin' flowers.

A recessive mutant producing heterocarpous flowers, with one (3%), two (82%) and three (15%) carpels and high fertility has been reported with a possibility of genetic engineering for fruit size, carpel number and seed number per plant (Rai and Kumar, 2001).

As periwinkle is also valued as an important garden plant because of its variously coloured flowers, mutants affecting flower colour, flower density, floral persistence etc., would also be of interest. A mutant described as 'leafless inflorescence', in which flowers are borne on nodes without leaves, has been reported and the locus '*lli*' has been mapped (Chaudhary *et al*., 2011). The mutant produced more number of flowers per plant than its parent and further enhancing its horticultural value. New improved horticultural genotypes were developed by crossing this mutant with other genotypes with different flower colours and plant habit (Kumar *et al*., 2012).

Another novel mutant with caducous closed corolla (corolla abscising before anthesis) inherited as a monogenic recessive, was isolated after mutagenesis with EMS (Supplementary Fig. 7S). The trait was used for development of cleistogamy, a new trait, in periwinkle (Kulkarni and Baskaran, 2013a).

Mutants with altered contents of alkaloids

There are not many studies where mutagenized populations have been screened for the contents of alkaloids. To the best of our knowledge, in only one study (Thamm, 2014), 3600 EMS mutagenized *C. roseus* plants were screened and one plant with high ajmalicine, and low catharanthine and vindoline contents was identified. RNA sequencing and comparative bioinformatics of mutant and wild-type plants showed up-regulation of SGD and the transcriptional repressor, Zinc finger Catharanthus transcription factor (*ZCT1*) in the mutant line. The increased SGD activity in mutants seemed to yield a larger pool of uncharacterized SGD reaction products that are channelled away from catharanthine and vindoline towards biosynthesis of ajmalicine when compared with the wild-type.

Mutants with tolerance to salt, water and heat stress

It is largely believed that plant secondary metabolites are synthesized in response to various kinds of abiotic and biotic stresses. Therefore, it would be of interest to study alkaloid accumulation in mutants exhibiting tolerance to

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

these stresses. Eight mutants (*gsr1* to *gsr8*), tolerant to salinity (250 mM NaCl) or high-temperature (45 °C) stress have been isolated (Rai *et al.*, 2001, 2003; Kumari *et al.*, 2013a, b). These mutants (*gsr 1* to *gsr 6*) accumulated more proline and glycine betaine constitutively as well as under water stress, and transpired lower amounts of water under water stress than their parental variety. The contents of catharanthine, VLB, VCR and serpentine in two of these *gsr* mutants (*gsr3* and *gsr6*) were found to be higher than those in their parental variety and correlated well with the expression profiles of TIA biosynthetic pathway genes, strictosidine synthase, desacetoxyvindoline 4-hydroxylase and deacdetyl vindoline 4-O-acetyl transferase (Dutta *et al.*, 2005). Although their most conspicuous mutant morphological traits were inherited as monogenic recessive traits, the mutants exhibited pleiotropic effects for several other traits (Rai *et al.*, 2003). Three of these mutations were thought to be in loci/genes that have very large and wide regulatory roles in the regulatory gene network of *C. roseus* for metabolism, development and adaptation to environment. The pleiotropies displayed by the mutants were considered to result from changes in gene expression affecting various kinds of functions responsible for achievement of plant morphology. The mutants were found to be hypomethylated at repeat sequences, up-regulated and down-regulated for many genes resulting in pleiotropic alterations for several traits (Kumari *et al.*, 2013a, b).

In recent years, mutations have been induced in Japan and China using high-energy (220 MeV) and low-energy (30 keV) ion beams, respectively, and many ornamental crop cultivars with unique colour characteristics have been developed. The spectrum of mutations induced by ion beam radiation was found to be different from that of gamma rays, and novel mutants have been identified in ornamental plants (Pathirana, 2011).

Space-flight environment has been found to induce mutations ('space breeding'). Spaceships/satellites have been used to expose seeds to space-flight environment. Both genetic and epigenetic changes and dominant mutations indicative of a different spectrum mutations have been detected in plants derived from seeds exposed to space flight (Yu *et al.*, 2007; Ou *et al.*, 2010). Use of these novel mutagens in periwinkle can, therefore, be expected to yield novel useful mutants. Desired level of specific metabolite may also be achieved *via* targeted mutagenesis (Belhaj *et al.*, 2013), as realized for reduced content of nornicotine in tobacco (Julio *et al.*, 2008). Saika *et al.* (2011) succeeded for the first time in producing a novel rice plant, with 230 times higher levels of tryptophan than normal plants, through site-targeted mutagenesis *via* gene targeting (GT) that could not have been obtained from conventional mutagenesis. They further suggested that it may be possible to achieve higher levels of various kinds of tryptophan-derived secondary metabolites, such as indole alkaloids, in plants obtained similarly from site-targeted mutagenesis *via* GT. Similarly, vindoline and catharanthine-rich mutants may be obtained if serpentine route gene functions can be knocked out (Singh *et al.*, 2008).

Detection of mutations is the first and most critical step in mutation breeding. Historically, mutants have been identified phenotypically, in large mutagenized populations, for easily recognizable characters such as, altered plant height and architecture, early or late flowering and maturity, altered flower, fruit and seed characteristics, resistance to diseases that can be screened easily in natural or artificial epiphytotics, and for biochemical quality traits for which low-cost, high-throughput and rapid evaluation methods are available. To increase efficiency of mutation breeding, high-throughput DNA technologies for mutation screening such as TILLING (Targeting Induced Limited Lesions IN Genomes), ECOTILLING, and high-resolution melt analysis (HRM), have been developed and used in crop plants (McCallum *et al.*, 2000; Comai *et al.*, 2004; Henikoff *et al.*, 2004; Mackay *et al.*, 2008; Xin *et al.*, 2008). These reverse genetics techniques can be used for discovering allelic variation in natural or mutagenized populations using large number of mutants isolated and TIA pathway genes already cloned in periwinkle.

Genomic resources, genetic linkage maps, quantitative trait loci (QTLs) and marker-assisted selection

There were no genomic resources or ESTs reported from this plant till 2006. Murata *et al.* (2006) sequenced two cDNA libraries from leaf base and root tips to generate 5023 ESTs of which 3553 could be annotated. This work served as the first platform to mine markers (microsatellite). With the rapid growing EST databases, efforts were initiated to develop molecular markers for *Catharanthus*. Medicinal plant genomic resource consortium presents 19899 ESTs clustering to 20460 contigs, 115 GSS and 749 nucleotides sequences (http://medicinalplantgenomics.msu.edu, as on 14th May, 2010). Currently 22867 ESTs, 88543 genomic sequences and 118 GSS and 46 transcriptome of *Catharanthus* are available in public domain (http://www.ncbi.nlm.nih.gov/date as on 19 December, 2014) which can be utilized for development of SSR, SNP and candidate gene markers. Comprehensive microsatellite and STMS marker resources (423) have been developed from genomic-enriched libraries (Shokeen *et al.*, 2005, 2007, 2011). A set of 350 unigene derived microsatellite markers of which 80 were found to be co-transferable to other Apocynaceses species such as *Rauwolfia serpentina, R. tetraphylla, R. vomitoria, Nerium* and *Tabernomontana* were developed (Jhang *et al.*, 2012). Similarly microsatellite

https://doi.org/10.1017/S1479262116000150 Published online by Cambridge University Press

marker resources from EST databases have been described (Joshi *et al*., 2011; Mishra *et al*., 2011). Transcriptome sequencing of 26 d old seedlings has been used recently to mine 2520 SSR markers of which a subset of 48 markers have been validated (Kumar *et al*., 2014).

The first framework genetic linkage map of *C. roseus* was constructed by Gupta *et al*. (2007). Six morphological markers and 125 DNA markers (79 RAPD, 7 ISSR, 2 EST-SSR, 37 other PCR-based DNA markers) mapped in the study resulted in 14 linkage groups generating a total map length of 1131.9 cM, with an average map length of 80.9 cM. The distance between two markers was 8.6 cM. Second genetic linkage map was developed using 423 co-dominant markers using 111 F_2 individuals derived from a cross CrN1 (Nirmal) × CrN82 (Kew), of which 134 were polymorphic between the parental genotypes. A total of 114 markers were mapped on eight linkage groups that spanned a 632.7 cM region of the genome with an average marker distance of 5.55 cM (Shokeen *et al*., 2011).

A genetic linkage framework map consisting of 172 DNA markers and one morphological marker (leaf-less inflorescence) was constructed by Chaudhary *et al*. (2011) and further advanced to include six additional markers (Sharma *et al*., 2012). Twenty QTLs for seven alkaloid yield related traits viz., contents of alkaloids in leaves, stems and roots, three harvest indices for leaves, stems and roots and total dry matter weight were identified and were found to be associated with five linkage groups, LG1, LG2, LG3, LG4 and LG6. Subsequently, they identified 20 QTLs, five for concentration of catharanthine in leaves, four each for concentrations of vindoline in leaves, catharanthine and serpentine in roots, two for ajmailicine in roots and 1 for VLB in leaves, located in four linkage groups, LG1, LG3, LG4 and LG6, by applying single marker analysis, simple interval mapping and composite interval mapping (Chaudhary *et al*., 2013). They further demonstrated the feasibility of marker-assisted selection for alkaloid yield related traits the first time in periwinkle. However, QTLs may not show the same effects in other genetic back grounds and environments and need to be validated. According to Brumlop and Finckh (2011), 'the more important a crop economically is, the more likely markers are applied in the breeding process', which aptly explains the late and slow developments in this area in periwinkle.

Conclusions

It is apparent from above review that periwinkle is an extensively investigated medicinal plant during the last half-a-century. As a result, it has emerged as a model 'non-model' plant for the study of alkaloid biosynthesis in plants. It is still the sole source of anti-cancer alkaloids, VLB and VCR, and its precursors, vindoline and catharanthine, in spite of extensive efforts to produce these alkaloids more economically *ex vivo*, through chemical synthesis, cell, tissue or organ cultures, or metabolic engineering than through extraction from field-grown plants.

Periwinkle is a diploid self-compatible, ever-blooming perennial plant species with a small chromosome number and a relatively short seed-to-seed cycle. It is thus ideally suited for genetic, cytogenetic research and for genetic enhancement through plant breeding. However, as it is a herkogamous and entomophyllus species, the need for pollination control (artificial self-pollination for advancing generations and maintenance of genetic purity), non-synchronous fruit maturity, dehiscent fruits, seed dormancy, short period of seed viability (about 12–18 months) and high susceptibility to die-back disease coupled with lack of availability of rapid methods for quantitative estimation of alkaloids amenable to plant breeding programmes appear to be some of the constraints in breeding of periwinkle.

In general, the genetic base of periwinkle populations from the Indian subcontinent and surrounding Asian region has been found to be narrow. Therefore, the need for germplasm collection from tropical and sub-tropical regions, particularly from its the centre of origin, cannot over emphasized. Mutation breeding using new mutagens, such as low- and high-energy ion beams and space-flight environment, could be explored as they have been reported to have induced higher frequency and a different spectrum of mutations (both genetic and epigenetic) than conventional mutagens.

A large number of loci are known to be involved in the production of lesion-mimic mutants in plants. As an induced lesion-mimic mutant in periwinkle exhibited high contents of leaf alkaloids, lesion-mimic mutants could used as early expressing morphological markers to identify mutants with high contents of alkaloids in populations derived after mutagen treatment. Double-mutant recombinants could be developed from non-allelic lesion-mimic mutants exhibiting enhanced contents of alkaloids to further enhance the alkaloid contents. Site-targeted mutagenesis *via* targeting genes involved in the secologanin pathway, considered to be the rate-limiting step in TIA production, could be attempted. The TILLING approach could be adopted to discover allelic variation in mutagenized populations using mutant libraries.

A beginning has just been made for marker-assisted selection for alkaloid content and yield by identification of QTLs for these traits. Validation of these QTLs in different genetic back grounds and their use for simultaneous improvement in contents and yields of economically important alkaloids could be expected in the near future.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1479262116000150.

Acknowledgements

The authors thank the Director CSIR-CIMAP, Lucknow, India for library and internet facilities.

References

Arens H, Stockigt J, Weiler EW and Zenk MH (1978) Radioimmunoassays for the determination of the indole alkaloids ajmalicine and serpentine in plants. *Planta Medica* 34: 37–46.

Baskaran K, Srinivas KVNS and Kulkarni RN (2013) Two induced macro-mutants of periwinkle with enhanced contents of leaf and root alkaloids and their inheritance. *Industrial Crops and Products* 43: 701–703.

Belhaj K, Chaparro-Garcia A, Kamoun S and Nekrasov V (2013) Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Methods* 9: 39.

Boke NH (1948) Development of perianth in *Vinca rosea* L. *American Journal of Botany* 35: 413–423.

Boke NH (1949) Development of the stamens and carpels in *Vinca rosea* L. *American Journal of Botany* 36: 535–547.

Bowman RN (2000) Method of producing hybrid *Catharanthus* using male sterility. US Patent 6,166,306 A, 26 December 2000.

Brown S, Clastre M, Courdavault V and O'Connor SE (2015) De novo production of the plant-derived alkaloid strictosidine in yeast. *Proceedings of the National Academy of Sciences of the United States of America* 112: 3205–3210. doi: 10.1073/pnas.1423555112.

Brumlop S and Finckh MR (2011) *Applications and Potentials of Marker Assisted Selection (MAS) in Plant Breeding*. Bonn, Germany: Federal Agency for Nature Conservation.

Bruneton J (1995) *Pharmacognosy, Phytochemistry, Medicinal Plants*. Paris: Lavoisier.

Campos-Tamayo F, Hernandez-Dominguez E, and Vazquez-Flota F (2008) Vindoline formation in shoot cultures of *Catharanthus roseus* is synchronously activated with morphogenesis through the last biosynthetic step. *Annals of Botany* 102: 409–415.

Chaudhary S, Sharma V, Prasad M, Bhatia S, Tripathi BN, Yadava G and Kumar S (2011) Characterization and genetic linkage mapping of the horticulturally important mutation leafless inflorescence (*lli*) in periwinkle *Catharanthus roseus*. *Scientia Horticulturae* 129: 142–153.

Chaudhary S, Pandey R, Sharma V, Tripathi BN and Kumar S (2013) Detection and mapping of QTLS affecting contents of pharmaceutical alkaloids in leaf and root of *Catharanthus roseus*. *Agricultural Research* 2: 9–23.

Comai L, Young K, Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR and Henikoff S (2004) Efficient discovery of nucleotide polymorphisms in populations by ECOTILLING. *The Plant Journal* 37: 778–786.

Cragg GM and Newman DJ (2005) Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology* 100: 72–79.

Dehghan E, Häkkinen ST, Oksman-Caldentey K-M and Ahmadi FS (2012) Production of tropane alkaloids in diploid and tetraploid plants and *in vitro* hairy root cultures of Egyptian henbane (*Hyoscyamus muticus* L.). *Plant Cell Tissue and Organ Culture* 110: 35–44.

De Padua DS, Barrion AA, Casal CMV and De La Cruz MaPR (1992) Karyomorphology of chichirica *Catharanthus roseus* (L.) Don. *Philippine Journal of Science* 121: 299–303.

Dhawan OP and Lavania UC (1996) Enhancing the productivity of secondary metabolites via induced polyploidy: a review. *Euphytica* 87: 81–89.

Dias DA, Urban S and Roessner U (2012) A historical overview of natural products in drug discovery. *Metabolites* 2: 303–336.

Di Fiore S, Li Q, Leech MJ, Schuster F, Emans N, Fischer R and Schillberg S (2002) Targeting tryptophan decarboxylase to selected subcellular compartments of tobacco plants affects enzyme stability and in vivo function and leads to a lesion mimic phenotype. *Plant Physiology* 129: 1160–1169.

Dnyansagar VR and Sudhakaran IV (1968) Meiotic Studies in *Vinca rosea* Linn. *Cytologia* 33: 453–464.

Dnyansagar VR and Sudhakaran IV (1970) Induced tetraploidy in *Vinca rosea* Linn. *Cytologia* 35: 227–241.

Dnyansagar VR and Sudhakaran IV (1977) Seed development in diploid and tetraploid of *Vinca rosea* syn. *Catharanthus roseus* (*Lochnera rosea*). *Proceedings of the National Academy of Sciences, India* 43 (Part B): 133–141.

Duge de Bernonville T, Clastre M, Besseau S, Oudin A, Burlat V, Glevarec G, Lanoue A, Papon N, Giglioli-Guivarc'h N, Benoit St-Pierre B and Courdavault V (2015) Phytochemical genomics of the Madagascar periwinkle: unravelling the last twists of the alkaloid engine. *Phytochemistry* 113: 9–23.

Dutta A, Batra J, Pandey-Rai S, Singh D, Kumar S and Sen J (2005) Expression of terpenoid indole alkaloid biosynthetic pathway genes corresponds to accumulation of related alkaloids in *Catharanthus roseus* (L.) Don. *Planta* 220: 376–383.

Dwivedi S, Singh M, Singh AP, Sharma S, Uniyal GC and Kumar S (1999) Genetic variability, heritability and genetic advance foralkaloid yield attributing traits in 26 genotypes of periwinkle *Catharanthus roseus*. *Journal of Medicinal and Aromatic Plant Sciences* 21: 320–324.

Dwivedi S, Singh M, Singh AP, Sharma S, Uniyal GC and Kumar S (2000) Assessment of genetic divergence for its purposeful exploitation in periwinkle *Catharanthus roseus* (Apocynaceae). *Journal of Genetics and Breeding* 54: 95–99.

El-Sayed M and Verpoorte R (2007) Catharanthus terpenoid indole alkaloids: biosynthesis and regulation. *Phytochemical Reviews* 6: 277–305.

Fabricant DS and Farnsworth NR (2001) The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives* 109 (Supplement 1): 69–75.

Facchini PJ and De Luca V (2008) Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. *Plant Journal* 54: 763–784.

Flory WS Jr (1944) Inheritance studies of flower colour in periwinkle. *Proceedings of American Society of Horticultural Science* 44: 525–526.

Goswami R, Tyagi BR, Rani A, Uniyal GC and Kumar S (1996) Colchicine induced autotetraploids in periwinkle *Catharanthus roseus*. *Journal of Medicinal and Aromatic Plant Sciences* 18: 38–45.

Guimarães G, Cardoso L, Oliveira H, Santos C, Duarte P and Sottomayor M (2012) Cytogenetic characterization and genome size of the medicinal plant *Catharanthus roseus* (L.) G. Don. *AoB PLANTS* 2012: pls002. doi: 10.1093/aobpla/pls002.

Guirimand G, Guihur A, Poutrain P, Héricourt F, Mahroug S, St-Pierre B, Burlat V, and Courdavault V (2011) Spatial organization of the vindoline biosynthetic pathway in *Catharanthus roseus*. *Journal of Plant Physiology* 168: 549–557.

Gupta S, Pandey-Rai S, Srivastava S, Naithani SC, Prasad M and Kumar S (2007) Construction of genetic linkage map of the medicinal and ornamental plant *Catharanthus roseus*. *Journal of Genetics* 86: 259–268.

Henikoff S, Till BJ and Comai L (2004) TILLING. Traditional mutagenesis meets functional genomics. *Plant Physiology* 135: 630–636.

Husain A (1993) *Medicinal Plants and their Cultivation*. Lucknow, India: Central Institute of Medicinal and Aromatic Plants.

Janaki Ammal EK and Bezbaruah HP (1963) Induced teteraploidy in *Catharanthus roseus* (L.) Don. *Proceedings of the National Academy of Sciences, India, Section B* 57: 339–342.

Jhang T, Gautam TP, Shukla S, Fatayal D, Annula and Kulkarni RN (2012) Development of microsatellite marker resource for genome analysis of *Catharanthus roseus*. In: *Proceedings of International Plant Conference on "Molecular Mapping & Marker Assisted Selection"*, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria. Vienna (8–11th February 2012) International Plant Conference Association, p 39.

Jia C-H, Dai Z-F, Xu B-Y, Jin Z-Q, Zhang L, Chen Y-Y and Wang J-B (2008) Analysis of karyotype of *Catharanthus roseus* (Apocynaceae). *Journal of Tropical and Subtropical Botany* 16: 169–172.

Johal GS, Hulbert SH, and Briggs SP (1995) Disease lesion mimics of maize: a model for cell death in plants. *Bioessays* 17: 685–692.

Joshi RK, Kar B and Nayak S (2011) Exploiting EST databases for the mining and characterization of short sequence repeat (SSR) markers in *Catharanthus roseus* L. *Bioinformation* 5: 378–81.

Julio E, Laporte F, Reis S, Rothan C and Dorlhac de Borne F (2008) Reducing the content of nornicotine in tobacco via targeted mutation breeding. *Molecular Breeding* 21: 369–381.

Knuth P (1909) *Handbook of Flower Pollination*, vol. III, Oxford: Clarendon Press.

Krishnan R (1995) Periwinkle. In: Chadda KL and Gupta R (eds) *Advances in Horticulture*, vol. 11. New Delhi: Malhotra Publishing House, pp. 409–428.

Krishnan R, Naragund VR and Vasantha Kumar T (1979) Evidence for outbreeding in *Catharanthus roseus*. *Current Science* 48: 80–82.

Krishnan R, Chandravadana MV, Mohan Kumar GN and Ramachander PR (1985) Effect of induced autotetrapoidy on alkaloid content and root weight in *Catharanthus roseus* (L.) G. Don. *Herba Hungarica* 24: 43–51.

Ku C, Chung W-C, Chen L-L and Kuo C-H (2013) The Complete plastid genome sequence of Madagascar periwinkle *Catharanthus roseus* (L.) G. Don: plastid genome evolution, molecular marker identification, and phylogenetic implications in Asterids. *PLoS ONE* 8: e68518. doi: 10.1371/journal pone.0068518

Kulkarni RN (1984) Relative resistance of diploid tetraploid plants of *Catharanthus roseus* plants to die back disease. *Current Science* 53: 577.

Kulkarni RN (1999) Evidence for phenotypic assortative mating for flower colour n periwinkle. *Plant Breeding* 118: 561–564.

Kulkarni RN and Baskaran K (2003) Inheritance of resistance to *Pythium* dieback in the medicinal plant, periwinkle. *Plant Breeding* 122: 184–187.

Kulkarni RN and Baskaran K (2008) Inheritance of pollen-less anthers and 'thrum' and 'pin' flowers in periwinkle. *Journal of Heredity* 99: 426–431.

Kulkarni RN and Baskaran K (2013a) From Herkogamy to Cleistogamy – development of cleistogamy in periwinkle. *Journal of Heredity* 104: 140–148.

Kulkarni RN and Baskaran K (2013b) Individual and combined effects of genes producing opposite effects on plant height in periwinkle (*Catharanthus roseus*). *Journal of Crop Science and Biotechnology* 16: 123–129.

Kulkarni RN and Baskaran K (2014) Increasing total leaf alkaloid concentrations in periwinkle (*Catharanthus roseus*) by combining the macro-mutant traits of two induced leaf mutants ('necrotic leaf' and 'nerium leaf'). *Journal of Horticultural Science and Biotechnology* 89: 513–518.

Kulkarni RN and Ravindra NS (1988) Resistance to *Pythium aphanidrmatum* in diploids and induced autotetraploids of *Catharanthus roseus*. *Planta Medica* 54: 356–359.

Kulkarni RN and Ravidra NS (1997) Integration of host resistance with fungicide in the control of dieback of periwinkle. *Tropical Agriculture* 74: 321–323.

Kulkarni RN, Chandrashekar RS and Dimri BP (1984) Induced autotetraploidy in *Catharanthus roseus*: a preliminary report. *Current Science* 53: 484–486.

Kulkarni RN, Rajagopal K, Chandrashekar RS, Dimri BP, Suresh N and Rao BRR (1987) Performance of diploids and induced autotetraploids of *Catharanthus roseus* under different levels of nitrogen and plant spacing. *Plant Breeding* 98: 136–140.

Kulkarni RN, Kalra A and Ravindra NS (1992) Integration of soil solarization with host resistance in the control of die back and collar rot of periwinkle. *Tropical Agriculture* 69: 217–222.

Kulkarni RN, Chandrashekara RS and Chadrashekara G (1995) Nitrogen utilization efficiency of diploid and induced autotetraploids strains of periwinkle. *Tropical Agriculture* 72: 249–251.

Kulkarni RN, Baskaran K, Chandrashekara RS and Kumar S (1999) Inheritance of morphological traits of periwinkle mutants with modified contents and yields of leaf and root alkaloids. *Plant Breeding* 118: 71–74.

Kulkarni RN, Sreevalli Y, Baskaran K and Kumar S (2001) The mechanism and inheritance of intraflower self-pollination in self-pollinating strains of periwinkle. *Plant Breeding* 120: 247–250.

Kulkarni RN, Sreevalli Y and Baskaran K (2005a) Allelic genes at two loci govern different mechanisms of intraflower self-pollination in self-pollinating strains of periwinkle. *Journal of Heredity* 95: 71–77.

Kulkarni RN, Baskaran K and Sreevalli Y (2005b) Genetics of novel corolla colours in periwinkle. *Euphytica* 144: 101–107.

Kulkarni RN, Baskaran K and Sreevalli Y (2008) Genetics of corolla colour in periwinkle: relationship between genes determining violet, orange-red and magenta corolla. *Journal of Applied Horticulture* 10: 20–23.

Kulkarni RN, Baskaran K, Shyamaprasad DV and Kulkarni SS (2009) Individual and combined effects of plant height reducing genes in periwinkle. *Euphytica* 170: 309–316.

Kumar S, Chaudhary S, Kumari R, Sharma V and Kumar AA (2012) Development of improved horticultural genotypes characterized by novel over-flowering inflorescence trait in periwinkle *Catharanthus roseus*. *Proceedings of the National Academy of Sciences, India, Section B, Biological Sciences* 82: 399–404.

Kumar S, Shah S, Garg V and Bhatia S (2014) Large scale in-silico identification and characterization of simple sequence repeats (SSRs) from *de novo* assembled transcriptome of *Catharanthus roseus* (L.) G. Don. *Plant Cell Reports* 33: 905–918.

Kumari R, Sharma V, Sharma V and Kumar S (2013a) Pleiotropic phenotypes of the salt-tolerant and cytosine hypomethylated leafless inflorescence, evergreen dwarf and irregular leaf

lamina mutants of *Catharanthus roseus* possessing Mendelian inheritance. *Journal of Genetics* 92: 369–394.

Kumari R, Yadav G, Sharma V, Sharma V and Kumar S (2013b) Cytosine hypomethylation at CHG and CHH sites in the pleiotropic mutants of Mendelian inheritance in *Catharanthus roseus*. *Journal of Genetics* 92: 499–511.

Lahlou M (2013) The success of natural products in drug discovery. *Pharmacology & Pharmacy* 4: 17–31.

Lavania UC (2005) Genomic and ploidy manipulation for enhanced production of phyto-pharmaceuticals. *Plant Genetic Resources: Characterization and Utilization* 3: 170–177.

Lavania UC, Srivastava S, Lavania S, Basu S, Misra NK and Mukai Y (2012) Autopolyploidy differentially influences body size in plants, but facilitates enhanced accumulation of secondary metabolites, causing increased cytosine methylation. *Plant Journal* 71: 539–549.

Levy A (1982) Natural and induced genetic variation in the biosynthesis of alkaloids and other secondary metabolites. In: *Improvement of Oil Seed and Industrial Crops by Induced Mutations*. Vienna, Austria: IAEA, pp. 213–222.

Levy A, Milo J, Ashri A and Palevitch D (1983) Heterosis and correlation analysis of the vegetative components and ajmalicine content in the roots of the medicinal plant – *Catharanthus roseus* (L.) G. Don. *Euphytica* 32: 557–564.

Lin X, Zhou Y, Zhang J, Lu X, Zhang F, Shen Q, Wu S, Chen Y, Wang T and Tang K (2011) Enhancement of artemisinin content in tetraploid *Artemisia annua* plants by modulating the expression of genes in artemisinin biosynthetic pathway. *Biotechnology and Applied Biochemistry* 58: 50–57.

Mackay JF, Wright CD and Bonfiglioli RG (2008) A new approach to varietal identification in plants by microsatellite high resolution melting analysis: application to the verification of grapevine and olive cultivars. *Plant Methods* 4: 8.

Madani H, Hosseini B, Dehghan E and Rezaei-chiyaneh E (2015) Enhanced production of scopolamine in induced autotetraploid plants of *Hyoscyamus reticulatus* L. *Acta Physiologiae Plantarum* 37: 55. doi: 10.1007/s11738–015–1795-x

Magnotta M, Murata J, Chen J and De Luca V (2006) Identification of a low vindoline accumulating cultivar of *Catharanthus roseus* (L.) G. Don by alkaloid and enzymatic profiling. *Phytochemistry* 67: 1758–1764.

Matsuura HN, Rau MR and Fett-Neto AG (2014) Oxidative stress and production of bioactive monoterpene indole alkaloids: biotechnological implications. *Biotechnology Letters* 36: 191–200.

Mba C (2013) Induced mutations unleash the potentials of plant genetic resources for food and agriculture. *Agronomy* 3: 200–231.

McCallum CM, Comai L, Greene EA and Henikoff S (2000) Targeted screening for induced mutations. *Nature Biotechnology* 18: 455–457.

Micke A (1988) Genetic improvement of grain legumes using induced mutations: an overview. In: *Improvement of Grain Legume Production using Induced Mutations*. Vienna, Austria: IAEA, pp. 1–51.

Miettinen K, Dong L, Navrot N, Schneider T, Burlat V, Pollier J, Woittiez L, van der Krol S, Lugan R, Ilc T, Verpoorte R, Oksman-Caldentey KM, Martinoia E, Bouwmeester H, Goossens A, Memelink J and Werck-Reichhart D (2014) The seco-iridoid pathway from *Catharanthus roseus*. *Nature Communications* 5, Article number: 3606. doi: 10.1038/ncomms4606.

Milo J, Levy A, Akavia N, Ashri A, and Palevitch D (1985) Inheritance of corolla colour and anthocyanin pigments in periwinkle *Catharanthus roseus* (L) G. Don. *Zeitschrift für Pflanzenzüchtung* 95: 352–360.

Mishra P and Kumar S (2001) A monogenic recessive mutant with precocious *in situ* pollen germination in periwinkle *Catharanthus roseus*. *Journal of Medicinal and Aromatic Plant Sciences* 22/4A &23/1A: 277–279.

Mishra P and Kumar S (2003) Manifestation of heterostyle character by induction of recessive *hsf* mutation responsible for thrum type herkogamous flowers in *Catharanthus roseus*. *Journal of Medicinal and Aromatic Plant Sciences* 25: 2–7.

Mishra P, Uniyal GC, Sharma S and Kumar S (2001) Pattern of diversity for morphological and alkaloid yield related traits among the periwinkle *Catharanthus roseus* accessions collected from in and around Indian subcontinent. *Genetic Resources and Crop Evolution* 48: 273–286.

Mishra RK, Gangadhar BH, Yu JW, Kim DH and Park SP (2011) Development and characterization of EST based SSR markers in Madagascar periwinkle (*Catharanthus roseus*) and their transferability in other medicinal plants. *Plant Omics Journal* 4: 154–162.

Mohan Kumar GN (1980) Comparative studies on growth and alkaloid of autotetraploids and their diploid progenitors in *Catharanthus roseus* (L.) G. Don. M. Sc. (Hort) Thesis, University of Agricultural Sciences, Bangalore, India.

Moreno PRH, van der Heijden R and Verpoorte R (1995) Cell and tissue cultures of *Catharanthus roseus* (L.) G. Don: a literature survey II. Updating from 1988 to 1993. *Plant Cell Tissue and Organ Culture* 42: 1–25.

Moudi M, Go R, Yien CYS, Nazre M (2013) Vinca alkaloids. *International Journal of Preventive Medicine* 4: 1231–1235.

Murata J, Bienzle D, Brandle JE, Sensen CW and De Luca V (2006) Expressed sequence tags from Madagascar periwinkle (*Catharanthus roseus*). *FEBS Letters* 580: 4501–4507.

Nef C, Rio B and Chrestin H (1991) Induction of catharanthine synthesis and stimulation of major indole alkaloids production by *Catharanthus roseus* cells under non-growth altering treatment with *Pythium vexans* extracts. *Plant Cell Reports* 10: 26–29.

Noble RL, Beer CT and Cutts JH (1958) Role of chance observations in chemotherapy: *Vinca rosea*. *Annals of the New York Academy of Sciences* 76: 882–894.

Ou X, Long L, Wu Y, Yu Y, Lin X, Qi X and Liu B (2010) Spaceflight-induced genetic and epigenetic changes in the rice (*Oryza sativa* L.) genome are independent of each other. *Genome* 53: 524–32.

Pareek SK, Singh S, Srivastava VK, Mandal S, Maheshwari ML and Gupta R (1981) Advances in periwinkle cultivation. *Indian Farming* 31: 18–21.

Palazon J, Cusido RM, Gonzalo J, Bonfill M, Morales C and Pinol MT (1998) Relation between the amount of *rolC* gene product and indole alkaloid accumulation in *Catharanthus roseus* transformed root cultures. *Journal of Plant Physiology* 153: 712–718.

Pasquali G, Porto DD and Fett-Neto AG (2006) Metabolic engineering of cell cultures versus whole plant complexity in production of bioactive monoterpene indole alkaloids: recent progress related to old dilemma. *Journal of Bioscience and Bioengineering* 101: 287–296.

Pathirana R (2011) Plant mutation breeding in agriculture. CAB Reviews: *Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 6: No. 032: doi: 10.1079/PAVSNNR20116032m.

Pezzuto JM (1997) Plant-derived anticancer agents. *Biochemical Pharmacology* 53: 121–133.

Rai SP and Kumar S (2001) Heterocarpus flowers resulting from a recessive mutation in periwinkle *Catharanthus roseus*. *Current Science* 80: 1581–1584.

Rai SP, Luthra R and Kumar S (2001) Differential expression of proteins in monogenic salt resistant mutants with and without thermal stress in periwinkle *Catharanthus roseus*. *Journal of Medicinal and Aromatic Plant Sciences* 22/4A &23/1A: 287–290.

Rai SP, Luthra R and Kumar S (2003) Salt-tolerant mutants in glycophytic salinity response (GSR) genes in *Catharanthus roseus*. *Theoretical and Applied Genetics* 106: 221–230.

Rani N and Kumar K (2011) Karyomorphological studies in the genus *Catharanthus*. *Indian Journal of Genetics and Plant Breeding* 71: 55–60.

Rendle AB (1971) *The Classification of Flowering Plants, II*. Cambridge: Cambridge University Press.

Roepke J, Wu M, Salim V, Thamm AMK, Murata J, Ploss K, Boland W and De Luca V (2010) Vinca drug components accumulate exclusively in leaf exudates of Madagascar periwinkle. *Proceedings of the National Academy of Sciences of the United States of America* 107: 15287–15292.

Ross IA (1999) *Medicinal Plants of the World: Chemical Constituents, Traditional and Modern Medicinal Uses*. New Jersey: Humana Press.

Roychowdhury R and Tah J (2013) Mutagenesis – a potential approach for crop improvement. In: Hakeem KR, Ahmad P and Ozturk M (eds) *Crop Improvement: New Approaches and Modern Techniques*. USA: Springer, pp. 149–187.

Saika H, Oikawa A, Matsuda F, Onodera H, Saito K, and Toki S (2011) Application of gene targeting to designed mutation breeding of high-tryptophan rice. *Plant Physiology* 156: 1269–1277.

Saiman MZ (2014) Terpenoids and terpenoid indole alkaloids in *Catharanthus roseus* cell suspension cultures. http://hdl.handle.net/1887/29812

Salim V and De Luca V (2013) Towards complete elucidation of monoterpene indole alkaloid biosynthesis pathway: *Catharanthus roseus* as a pioneer system. In: Giglioli-Guivarc'h N (ed.) *Advances in Botanical Research Volume 68,* New Light on Alkaloid Biosynthesis and Future Prospects. Amsterdam, The Netherlands: Academic Press, pp. 1–37.

Salim AA, Chin Y-W and Kinghorn AD (2008) Drug discovery from plants. In: Ramawat KG, Merillon JM (eds) *Bioactive Molecules and Medicinal Plants*. New York: Springer, pp. 1–25.

Schippmann U, Cunningham AB and Leaman DJ (2003) Impact of cultivation and gathering of medicinal plants on biodiversity: global trends and issues. In: *Biodiversity and the Ecosystem Approach in Agriculture, Forestry and Fisheries Satellite Event on the Occasion of the Ninth Regular Session of the Commission on Genetic Resources for Food and Agriculture*. Rome 12–13 October 2002, pp. 141–167. URL: http://www.fao.org/docrep/005/y4586e/y4586e00.htm

Schmelzer GH (2007) *Catharanthus roseus* (L.) G.Don. In: Schmelzer GH and Gurib-Fakim A (eds) Prota 11(1): Medicinal plants/Plantes médicinales 1. [CD-Rom]. PROTA, Wageningen, The Netherlands. URL: http://database.prota.org/PROTAhtml/Catharanthus%20roseus_En.htm

Sevestre-Rigouzzo M, Nef-Campa C, Ghesquiere A and Chrestin H (1993) Genetic diversity and alkaloid production in *Catharanthus roseus, C. trichophyllus* and their hybrids. *Euphyitca* 66: 151–159.

Sharma V, Chaudhary S, Srivastava S, Pandey R and Kumar S (2012) Characterization of variation and quantitative trait loci related to terpenoid indole alkaloid yield in a recombinant inbred line mapping population of *Catharanthus roseus*. *Journal of Genetics* 91: 49–69.

Shokeen B, Sethy NK, Choudhary S and Bhatia S (2005) Development of STMS markers from the medicinal plant Madagascar periwinkle [*Catharanthus roseus* (L.) G. Don.]. *Molecular Ecology Notes* 5: 818–820.

Shokeen B, Sethy NK, Kumar S and Bhatia S (2007) Isolation and characterization of microsatellite markers for analysis of molecular variation in the medicinal plant Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don). *Plant Science* 172: 441–451.

Shokeen B, Choudhary S, Sethy NK and Bhatia S (2011) Development of SSR and gene-targeted markers for construction of a framework linkage map of *Catharanthus roseus*. *Annals of Botany* 108: 321–336.

Simmonds NW (1960) Flower colour in *Lochnera rosea*. *Heredity* 14: 253–261.

Singh J (1996) Ajmalicine (Raubasine): a medicinally important alkaloid from *Catharanthus roseus* (*Vinca rosea*). In: Handa SS and Kaul MK (eds) *Supplement to Cultivation and Utilization of Medicinal Plants*. Jammu-Tawi, India: Regional Research Laboratory, pp. 199–206.

Singh D, Rai SK, Pandey-Rai S, Srivastava S, Mishra K, Sharma S and Kumar S (2008) Predominance of the serpentine route in monoterpenoid indole alkaloid pathway of *Catharanthus roseus*. *Proceedings of Indian National Science Academy* 74: 97–109.

Snoeijer W (2001) *International Register of Catharanthus roseus*. Leiden: Leiden/Amsterdam Centre for Drug Research, Division of Pharmacognosy.

Sreevalli Y (2002) Inheritance of some morphological, floral, reproductive and economically important traits in the medicinal plant, periwinkle [*Catharanthus roseus* (L.) G. Don]. Ph.D. Thesis, Department of Botany, Bangalore, India.

Sreevalli Y, Baskaran K, Kulkarni RN and Kumar S (2000) Further evidence for the absence of automatic and intra-flower self-pollination in periwinkle. *Current Science* 79: 1648–1649.

Sreevalli Y, Kulkarni RN and Baskaran K (2002) Inheritance of flower colour in periwinkle: orange-red corolla and white eye. *Journal of Heredity* 93: 55–58.

Sreevalli Y, Baskaran K, and Kulkarni RN (2003) Inheritance of functional male sterility in the medicinal plant periwinkle. *Indian Journal of Genetics and Plant Breeding* 63: 365–366.

Stearn WT (1975) A synopsis of the genus *Catharanthus* (Apocynaceae). In: Taylor RW and Farnsworth NR (eds) *The Catharanthus Alkaloids. Botany, Chemistry, Pharmacology, and Clinical Use*. New York: Marcel Dekkar, pp. 9–44.

Svoboda GH (1975) Introduction. In: Taylor RW and Farnsworth NR (eds) *The Catharanthus Alkaloids. Botany, Chemistry, Pharmacology, and Clinical Use*. New York: Marcel Dekkar, pp. 1–7.

Svoboda GH and Blake DA (1975) The phytochemistry and pharmacology of *Catharanthus roseus* (L.) G. Don. In: Taylor RW and Farnsworth NR (eds) *The Catharanthus Alkaloids. Botany, Chemistry, Pharmacology, and Clinical Use*. New York: Marcel Dekkar, pp. 45–83.

Swaminathan MS (1972) Mutational reconstruction of crop ideotypes. In: *Induced Mutations and Plant Improvement*. Vienna, Austria: IAEA, pp. 155–170.

Thamm NK (2014) Induction and characterization of *Catharanthus roseus* mutant altered in monoterpenoid indole alkaloid biosynthesis. http://hdl.handle.net/10464/5682

Tyler VE (1988) Medicinal plant research: 1953–1987. *Planta Medica* 54: 95–100.

Virk SS, Singh OS and Bhullar BS (1988) Assessment of variability for different quantitative characters in periwinkle. *Crop Improvement* 15: 138–141.

van der Heijden R, Verpoorte R and Ten Hoopen HJG (1989) Cell and tissue cultures of *Catharanthus roseus* (L.) G. Don: a literature survey. *Plant Cell Tissue and Organ Culture* 18: 231–280.

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D and Verpoorte R (2004) The *Catharanthus* Alkaloids: Pharmacognosy and Biotechnology. *Current Medicinal Chemistry* 11: 1241–1253.

Verma P, Mathur AK, Srivastava A and Mathur A (2011) Emerging trends in research on spatial and temporal organization of terpenoid indole alkaloid pathway in *Catharanthus roseus*: a literature update. *Protoplasma* 249: 255–268.

Wang Y and Li J (2006) Genes controlling plant architecture. *Current Opinion in Biotechnology* 17: 123–129.

Wink M, Alfermann AW, Franke R, Wetterauer B, Distl M, Windhövel J, Krohn O, Fuss E, Garden H, Mohagheghzadeh A, Wildi E and Ripplinger P (2005) Sustainable bioproduction of phytochemicals by plant *in vitro* cultures: anticancer agents. *Plant Genetic Resources: Characterization and Utilization* 3: 90–100.

Wu C, Bordeos A, Madamba RS, Baraoidan M, Ramos M, Wang GL, Leach JE and Leung H (2008) Rice lesion mimic mutants with enhanced resistance to diseases. *Molecular Genetics and Genomics* 279: 605–619.

Xin Z, Wang ML, Barkley NA, Burow G, Franks C, Pederson G and Burke J (2008) Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in chemically induced sorghum mutant population. *BMC Plant Biology* 8: 103. doi: 10.1186/147-2229-8-103.

Xing S-H, Guo X-B, Wang Q, Pan Q-F, Tian Y-S, Liu P, Zhao J-Y, Wang G-F, Sun X-F and Tang K-X (2011) Induction and flow cytometry identification of tetraploids from seed-derived explants through colchicine treatments in *Catharanthus roseus* (L.) G. Don. *Journal of Biomedicine and Biotechnology* 2011, Article ID 793198, http://dx.doi.org/10.1155/2011/793198.

Yu F and De Luca V (2013) ATP-binding cassette transporter controls leaf surface secretion of anticancer drug components in *Catharanthus roseus*. *Proceedings of the National Academy of Sciences of the United States of America* 110: 15830–15835.

Yu X, Wu H, Wei LJ, Cheng ZL, Xin P, Huang CL, Zhang KP and Sun YQ (2007) Characteristics of phenotype and genetic mutations in rice after spaceflight. *Advances in Space Research* 40: 528–534.

Zhao J and Verpoorte R (2007) Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. *Phytochemical Reviews* 6: 435–457.

Zhao J, Hu Q, Guo Y-Q and Zhu WH (2001) Effects of stress factors, bioregulators, and synthetic precursors on indole alkaloid production in compact callus clusters cultures of *Catharanthus roseus*. *Applied Microbiology and Biotechnology* 55: 693–698.

Zhao L, Sander GW and Shanks JV (2013) Perspectives of the metabolic engineering of terpenoid indole alkaloids in *Catharanthus roseus* hairy roots. *Advances in Biochemical Engineering/Biotechnology* 134: 23–54.

Za´rate R and Verpoorte R (2007) Strategies for the genetic modification of the medicinal plant *Catharanthus roseus* (L.) G. Don. *Phytochemical Reviews* 6: 475–491.

Zhou M-L, Shao J-R and Tang Y-X (2009) Production and metabolic engineering of terpenoid indole alkaloids in cell cultures of the medicinal plant *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle). *Biotechnology and Applied Biochemistry* 52: 313–323.

Zhu X, Zeng X, Sun C and Chen S (2014) Biosynthetic pathway of terpenoid indole alkaloids in *Catharanthus roseus*. *Frontiers of Medicine* 8: 285–293.

Zonneveld BJM, Leitch IJ and Bennett MD (2005) First nuclear DNA amount in more than 300 Angiosperms. *Annals of Botany* 96: 229–244.