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RESEARCH NOTE

Silver nitrate-induced in vitro shoot multiplication and precocious flowering in *Catharanthus roseus* (L.) G. Don, a rich source of terpenoid indole alkaloids

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Abstract

Catharanthus roseus (L.) G. Don is an economically and medicinally important plant since its leaves and flowers contain terpenoid indole alkaloids. The present study, for the first time, encompasses the influence of silver nitrate ($AgNO_3$), in consort with cytokinins like N^6-benzyladenine (BA) and 6-furfurylaminopurine (kinetin), to regenerate multiple shoots from nodal segments explants and to induce high-frequency precocious flowering of *C. roseus* under in vitro condition. Synergistic effect of equal concentrations of BA and kinetin was enhanced following the amalgamation of $AgNO_3$. As high as 98% explants responded to multiple shoot initiation and proliferation in Murashige and Skoog medium supplemented with 3 µM BA, 3 µM kinetin and 0.1 µM $AgNO_3$. As many as 7 shoots were developed per explant following 12 days of inoculation. Continuous culture in the same medium for 21 days induced precocious flowering from 75% shoots, wherein a maximum of ~6 (5.67 ± 0.88) flowers was observed per in vitro shoot. On the other hand, in the combinations of BA and kinetin excluding $AgNO_3$, a maximum of 6.67% explants responded and initiated merely 3.33 shoots per explant. Nevertheless, no induction of flower was observed in the media devoid of $AgNO_3$. Our results on the induction and proliferation of multiple shoots with simultaneous flowering would help the global pharmaceutical industry to produce in vitro shoots and flowers in bulk, as an alternative source of alkaloids.

Keywords $AgNO_3$ · *Catharanthus* · In vitro flowering · Periwinkle · Multiple shoot proliferation

Abbreviations

$AgNO_3$	Silver nitrate
BA	N^6-benzyladenine
Kinetin	6-Furfurylaminopurine
MS	Murashige and Skoog (1962)
PGR	Plant growth regulator

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Catharanthus roseus (L.) G. Don (syn. *Vinca rosea* L.), a distinguished member of Apocynaceae family, is universally known as 'Madagascar periwinkle' (Łata 2007). Typically three varieties of *C. roseus*: the pink-flowered 'rosea', the white-flowered 'alba' and the white with a pink or yellow ring in the orifice region 'Ocellata' are commonly found depending on its flower colour. Thus shrub is a native of Western Indian Ocean's large island of Madagascar neighbouring to Africa and geographically distributed in the United States, Africa, Australia, China, India, Spain, and Southern Europe where it is grown widely for medicinal purposes. Even it prefers to grow on sandy soil beside coastland, yet can be frequently found on riverside, in savanna vegetation, in arid uncultivated areas and pavements, seldom in open forest or on rocky soils (Barkat et al. 2017). Owing to the presence of an extensive range of terpenoid indole alkaloids (catharanthine, vindoline, vincristine, and vinblastine) in its leaves and flowers (Srivastava et al. 2014), the potential medicinal value of *C. roseus* is gradually escalating in the global pharmaceutical industry. These compounds have a broad application in the treatment of Wilkins's tumour,

leukaemia in children, neuroblastoma and lymphocytic leukaemia, Hodgkin's disease and reticulum cell sarcoma, besides choriocarcinoma and lymphosarcoma. The dimeric alkaloids, such as vincristine and vinblastine produced from *C. roseus* leaves have a potential role against cancer (Barkat et al. 2017). Nevertheless, these two alkaloids are found in trace amount in the plants (Pietrosiuk et al. 2007). The roots of this plant were shown to have anti-hypertension alkaloid ajmalicine (Carolyn et al. 2005; Jaleel et al. 2006) whilst the dried or wet flowers were treated as a paste in some rural communities and also reported to have wound healing activities (Nayak and Pereira 2006). Considering the aforementioned pharmaceutical properties, the leaves, roots, and flowers are harvested incessantly over the decades that lead to the rapid depletion of the natural population of this plant. To avoid the overexploitation and increase the availability of these crucial alkaloids this plant needs accelerated propagation in mass. Tissue culture is the most efficient and reliable technique for mass multiplication within a short span of time (Gantait et al. 2015; Panigrahi et al. 2017). Additionally, in vitro plant cell culture, callus culture and multiple shoot cultures of *C. roseus* hold the potential of indole alkaloid (ajmalicine, catharanthine, vinblastine, vincristine, and vindoline) production in commercial scale (Satdive et al. 2003; Pietrosiuk et al. 2007; Pati et al. 2011; Almagro et al. 2014; Verma et al. 2015). Mass propagation of *C. roseus* in terms of in vitro multiple shoots and root culture, callus-mediated regeneration and somatic embryogenesis using different explants such as leaf, hypocotyl, epicotyl, nodal segments and roots was reported earlier (reviewed by Pietrosiuk et al. 2007). Yet, there is scanty of a report on induction of in vitro flower that can be exploited as an important and alternative source of alkaloids. There are several factors that were studied to induce precocious in vitro flowering such as, light intensity (Jumin and Nito 1996; Gantait et al. 2012; Jeong and Sivanesan 2015), media pH (Jumin and Ahmad 1999; Gantait et al. 2012), sucrose (Jeong and Sivanesan 2015), temperature, and plant growth regulators (PGRs) (Wang et al. 2002; Gantait et al. 2012; Jeong and Sivanesan 2015). $AgNO_3$ proved to be an effective elicitor to promote in vitro shoot regeneration of several cereal, oilseed and vegetable crops (Williams et al. 1990; Mohiuddin et al. 1997; Wu et al. 2006). However, till date, there was no such report on the influence of such an effective elicitor ($AgNO_3$) on in vitro flowering in *C. roseus*. On this backdrop, the present study was the first attempt to assess the influence of $AgNO_3$, in consort with two different cytokinins, to regenerate in vitro multiple shoots and to induce precocious flowering of *C. roseus*.

Healthy nodal segment explants of *C. roseus* (white variety 'alba') were harvested from 2-month-old seed germinated plants (during their non-flowering season) growing in the medicinal plant garden of Shri A.N. Patel Post Graduate Institute of Science and Research, Anand, Gujarat (22°33′14.8″N 72°57′38.2″E).

The explants were cleaned by rinsing thoroughly with running tap water for 2–3 times and then soaked with H_2O_2 for 2 min. They were then transferred to another beaker containing 2% (w/v) Bavistin with 2–3 drops of Tween-20 for 20 min and then treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 2 min. The explants were rinsed thrice with sterile water following each of the above steps.

Explants were inoculated in full strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with a combination of equal concentrations (1–5 µM) of N^6-benzyladenine (BA) and 6-furfurylaminopurine (kinetin). The cytokinin combinations were employed with and without 0.1 µM $AgNO_3$. All the in vitro cultures were incubated in the culture condition described below.

The MS medium, comprising 3% (*w*/*v*) sucrose and 100 mg/l myo-inositol was used. PGRs (SRL Chemicals, Mumbai, India) were added to the medium as indicated in above. MS medium without PGR or $AgNO_3$ was considered as the control. Prior addition of agar (7%; *w*/*v*), pH of the medium was attuned to 5.7 with 1 N NaOH or HCl. Media were then sterilized in an autoclave (A One, India) at 121 °C and 1 kg/cm^2 for 20 min. Subsequent to the inoculation of the explants, the cultures were incubated at 25 ± 1 °C under a 12 h photoperiod, with cool white fluorescent light (Phillips Life Max, India) with an irradiance of 40 µmol/m^2/s photosynthetic photon flux density (measured with LI-COR, Lincoln, USA).

Data on frequency of shoot induction (%) (number of explants inducing shoots out of 100), number of shoots per explant and days taken to reach highest response (i.e. maximum number of shoots) [for the assessment of in vitro multiple shoot proliferation], as well as frequency of flower induction (%) (number of shoots inducing flowers out of 100), days to flower induction and number of flowers per shoot [for the assessment of in vitro flower induction] were recorded.

A Completely Randomized Design was followed for the said experiment and the experiment was replicated thrice involving 20 samples per replication. Each one of the explants was considered as an experimental unit. The recorded data were statistically analyzed using SPSS (version 17.0, SPSS Inc., Chicago, IL, USA) software. Analysis of variance (ANOVA) was used to calculate statistical significance. Treatment data (mean ± standard error) differing significantly were assessed through the Duncan's multiple range test at $P = 0.05$ level (Duncan 1955).

In the present study, the control medium (without PGRs and $AgNO_3$) did not show any response towards the in vitro multiple shoot formation, but all the combinations of BA and kinetin initiated multiple shoots albeit with very low (2–6.67%) frequency. The maximum percent response

was observed by the combination of 5 µM BA and 5 µM kinetin, which was a meagre 6.67% after ~33 (32.67) days (Table 1). In contrast to the present study, the combination of BA and kinetin significantly boosted the mass propagation of *Hemidesmus* species (Patnaik and Debata 1996) and *Bauhinia racemosa* Lam. (Sharma et al. 2017) via multiple shoot initiation and proliferation. Such poor regeneration of in vitro shoot production in the present study may be attributed to the production of ethylene (Mohiuddin et al. 1997). The cytokinin such as BA and kinetin are often preferred alone (Bakrudden et al. 2011; Pati et al. 2011) but not in combination to initiate in vitro shoot initiation and multiplication of *C. roseus*. It also envisages that the cytokinin is meant for shoot regeneration. But, this concept was not proved substantially in the present study. Few other studies reported that the use of BA alone was more effective than in combination with kinetin during proliferation and multiplication of axillary buds in other medicinal plant (Sangeetha and Venkatachalam 2014) but, elongation of in vitro shoots was comparatively more frequent (with a longer internodes) in MS media supplemented with kinetin (Patnaik and Debata 1996; Bhattacharya and Bhattacharyya 1997; Bakrudden et al. 2011). On the other hand, in the present study, the fortification of $AgNO_3$ in the combinations of BA and kinetin resulted in a significant positive impact on the development of in vitro multiple shoots and their subsequent proliferation (Fig. 1a, b). Since the $AgNO_3$ concentration was fixed at 0.1 µM, the impact of the BA and kinetin combinations along with $AgNO_3$ was quite evident on in vitro multiple shooting. The influence of these combinations was enhanced with the increasing concentrations of BA and kinetin combinations, which displayed their best effect (98% response) at 3 µM and then gradually declined (Table 1). The best response with 7 shoots per explant was recorded within 12 days of inoculation in the same medium (3 µM BA plus 3 µM kinetin with 0.1 µM $AgNO_3$) (Fig. 1c). It implies that this was the best among all the treated combination. Alternatively, the higher concentrations of this combination resulted in reduced and delayed response with fewer shoots. This inductive effect of $AgNO_3$ corroborates the earlier observation on in vitro shoot regeneration of *Helianthus annus, Brassica oleracea, Triticum aestivum, Nicotiana plumaginifolia*, and such effect could be considered as the inhibitory attribute of $AgNO_3$ over ethylene (Purnhauser et al. 1987; Williams et al. 1990; Khalid et al. 1991).

During the continuous in vitro proliferation of the multiple shoots in the same medium, explants inoculated in combinations of BA or kinetin did not induce any flower at all. However, the similar combinations supplemented with 0.1 µl of $AgNO_3$ induced flower buds (Fig. 1e) that turned into completely bloomed flowers subsequently. The morphology of the in vitro induced flowers was similar to that of the natural in vivo flowers (Fig. 1f). Though each of the combination of BA and kinetin along with $AgNO_3$ responded to precocious flowering and the frequency of flowering ranged from a minimum of 16% to a maximum of 75%. The synergistic influence of $AgNO_3$ with BA and kinetin combinations displayed a surge in flower induction with their increasing levels, which recorded as high as 75% response at 3 µM and then subsequently dropped (Table 1). The most effective flowering was recorded after 21 (21.33 ± 0.67) days of culture with ~6 (5.67 ± 0.88 flowers per plant) in 3 µM

Table 1 Influence of plant growth regulators and silver nitrate ($AgNO_3$) on in vitro multiple shoots formation and flower induction in *Catharanthus roseus* (L.) G. Don

Plant growth regulators (µM)		$AgNO_3$ (µM)	Morphogenetic response					
BA	Kinetin		Frequency of shoot induction (%)	No. of shoots/explant	Days to highest response	Frequency of flower induction (%)	Days to flower induction	No. of flowers / shoot
0	0	0	0.00 ± 0.00j	0.00 ± 0.00d	0.00 ± 0.00i	0.00 ± 0.00e	0.00 ± 0.00f	0.00 ± 0.00c
1	1	0	2.00 ± 0.57i	2.33 ± 0.33c	39.00 ± 0.58a	0.00 ± 0.00e	0.00 ± 0.00f	0.00 ± 0.00c
2	2	0	3.33 ± 0.88hi	2.67 ± 0.67c	35.33 ± 0.33b	0.00 ± 0.00e	0.00 ± 0.00f	0.00 ± 0.00c
3	3	0	4.33 ± 0.33gh	3.33 ± 0.33bc	28.67 ± 0.67d	0.00 ± 0.00e	0.00 ± 0.00f	0.00 ± 0.00c
4	4	0	5.67 ± 0.33fg	2.33 ± 0.33c	30.67 ± 0.33cd	0.00 ± 0.00e	0.00 ± 0.00f	0.00 ± 0.00c
5	5	0	6.67 ± 0.33f	2.33 ± 0.33c	32.67 ± 0.33c	0.00 ± 0.00e	0.00 ± 0.00f	0.00 ± 0.00c
1	1	0.1	13.00 ± 1.53e	2.67 ± 0.67c	23.67 ± 0.88e	16.00 ± 1.00d	47.33 ± 1.45a	1.33 ± 0.33b
2	2	0.1	49.00 ± 1.00d	4.67 ± 0.88b	18.67 ± 0.67f	17.33 ± 0.88d	42.67 ± 0.88b	1.67 ± 0.33b
3	3	0.1	98.00 ± 1.00a	7.00 ± 0.58a	12.00 ± 1.15h	75.00 ± 1.53a	21.33 ± 0.67e	5.67 ± 0.88a
4	4	0.1	90.0 ± 0.00b	5.00 ± 0.58b	16.00 ± 1.00g	56.67 ± 1.45b	29.67 ± 1.20d	2.33 ± 0.67b
5	5	0.1	80.0 ± 0.00c	3.33 ± 0.67bc	19.33 ± 1.20f	39.00 ± 1.73c	32.00 ± 1.15c	2.33 ± 0.33b

The data represents mean ± standard error. Data for each column followed by the different alphabets are significantly different according to Duncan's multiple range test (Duncan 1955) at $p < 0.05$

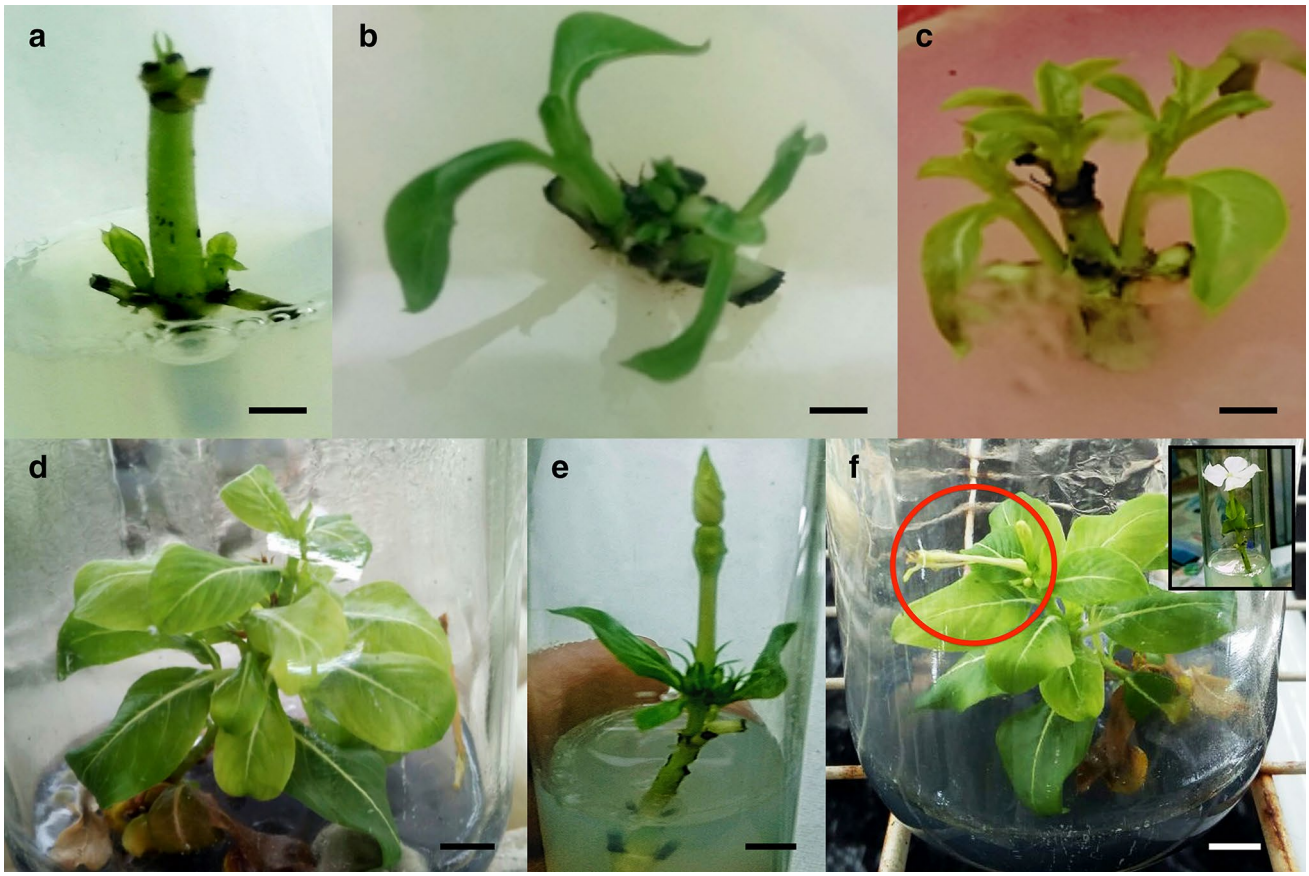

Fig. 1 In vitro multiple shoot formation and flower induction in *Catharanthus roseus* (L.) G. Don. **a** Initiation of axillary shoot buds from nodal segments (Bar = 3 mm), **b** elongation on shoots (Bar = 3 mm), **c** multiplication and proliferation of shoots (Bar = 5 mm), **d** well developed shoot with multiple leaves (Bar = 10 mm), **e** induction of flower buds (Bar = 10 mm), **f** induction development of multiple flower buds (marked in red circle) (inset: an in vitro flower in full bloom) (Bar = 10 mm)

BA plus 3 µM kinetin with 0.1 µM $AgNO_3$ (Fig. 1d). On the other hand, the higher concentrations of this combination resulted in delayed (~ 30–32 days) and lower flowering frequencies (~ 39–57%) with more or less two flowers per plant (Table 1). In an earlier study, it was found that only BA at 13.3 µM or kinetin at 13.9 µM resulted in the breaking of maximum axillary shoot buds (but not flower bud) following 45 long days of culture in *C. roseus* (Bakrudden et al. 2011). The use of an in vitro system to study flowering allows the control and assessment of a wide range physicochemical factors. Some of the reports suggested that in vitro flowering depends on physical factors such as photoperiodism, temperature; and/or application of PGRs like GA_3 also influenced in vitro flowering (Gantait et al. 2012; Jeong and Sivanesan 2015). But, the present study affirmed $AgNO_3$ as the only elicitor (in supplementation with BA-kinetin combination) that induced multiple shoots and flowers. Such influence of $AgNO_3$ on in vitro flowering might be due to the enhancement of the pool of endogenous polyamines and initially improved the shoot regeneration and later on flowering. This result was well supported by Bais et al. (2000).

The present study investigated an alternative and unique approach to induce multiple shoots and simultaneous precocious flowering in *C. roseus*; a novel technique from which the global pharmaceutical industry would immensely be benefitted by the producing in vitro multiple shoots and flowers (the rich source of alkaloids) in large scale. Additionally, the present study would seem to explore advanced opportunities for in vitro breeding of *C. roseus*.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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