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Research note

Inorganic source of selenium in the culture of calluses of *Catharanthus roseus* (L.)

Luis Guillermo Terreros-Rosales[1]
José Juvencio Castañeda-Nava[1]
Janet María León-Morales[2]
Soledad García-Morales[3,§]

1 Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco. Camino Arenero núm. 1227, El Bajío, Zapopan, Jalisco, México. CP. 45019. Tel. 333 3455200, ext. 2032. (luterreros-al@ciatej.edu.mx; jcastaneda@ciatej.mx).

2 Coordinación Académica-Región Altiplano Oeste-Universidad Autónoma de San Luis Potosí. Carretera Salinas-Santo Domingo núm. 200, Salinas de Hidalgo, San Luis Potosí, México. CP. 78600. Tel. 496 9634030. (janet.leon@uaslp.mx).

3 CONAHCYT-Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco. Camino Arenero núm. 1227, El Bajío, Zapopan, Jalisco, México. CP. 45019. Tel. 333 3455200, ext. 2033.

Autora para correspondencia: smorales@ciatej.mx

Abstract

Catharanthus roseus (L.) is a species with high pharmaceutical and ornamental value, which has led to the search for biotechnological alternatives to improve the establishment of cell cultures. This work aimed to induce callus formation in different explants of *C. roseus* and to evaluate the effect of two inorganic sources of selenium (Se) on their growth in order to identify the best range of concentrations. Callus formation was induced from hypocotyls and leaves, evaluating three different culture media. To increase the biomass production of *C. roseus* calluses, sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4) were used as inorganic sources of Se. Callus induction and growth assessment were performed in 2022. Concentrations of 1, 5, 10, 15, 20, 50, and 100 mg L^{-1} of both compounds were tested in two independent experiments. Treatments with 1, 5, and 10 mg L^{-1} of Na_2SeO_3 and Na_2SeO_4 produced friable calluses with higher fresh weight. The source of Se influenced the growth of calluses; a fresh weight of 0.52 g was obtained with 1 mg L^{-1} of Na_2SeO_4, higher than that obtained with the same concentration of Na_2SeO_3. Concentrations greater than 20 mg L^{-1} from both sources negatively affected callus growth.

Keywords:

Catharanthus roseus, callogenesis, selenate, selenite.

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Catharanthus roseus, also known as vinca, is a perennial herbaceous species with a high ornamental and cultural value due to its use in traditional medicine. This species is used in different cultures worldwide to treat various ailments such as stomach infections, gastritis, or asthma. Leaf and root extracts have also been reported to have anticancer, antioxidant, anti-inflammatory, and antimicrobial activities (Das *et al*., 2020).

In addition, several alkaloids produced in this species, such as ajmalicine, vinblastine, and vincristine, have anticancer activities (Dhyani *et al*., 2022). There are pharmaceutical products from these compounds that generate more than 100 million dollars per year (Rojas-Sandoval, 2022). Thus, the study of *C. roseus* has gained great importance in scientific research focused on the production of drugs that involve the secondary metabolites produced by this species (Dhyani *et al*., 2022).

Although this plant has high yields in seed reproduction, asexual propagation is necessary to ensure less genetic variability and greater homogeneity of plant material in large-scale production. It is necessary to standardize crop conditions such as temperature and photoperiod in regions where species do not grow naturally and concentrations and types of growth regulators according to plant species (Salas-Valdivia *et al*., 2023).

Nonetheless, traditional propagation is not recommended for large-scale production due to low multiplication rates (Rojas-Sandoval, 2022). Over the past few decades, *in vitro* culture technologies have been explored in *C. roseus*, where callus production stands out (Rahman *et al*., 2019).

On the other hand, calluses have been studied in plants due to their positive effects on different plant species. In the culture of *C. roseus* suspension cells, a higher amount of alkaloids, such as ajmalicine and serpentine, was produced when the growth medium had some source of Se (Na_2SeO_3 or Na_2SeO_4), compared to the control (Arvy *et al*., 1995). Na_2SeO_3 improved the growth of *Calendula officinalis* (L.), increasing seedling height, leaf area, and number of leaves (Hernández-Díaz *et al*., 2021). Se, in the form of nanoparticles, had a biostimulant effect on the development and yield of *C. roseus* biomass and *C. officinalis* flowers (Garza-García *et al*., 2023).

The different sources of Se are associated with changes in plant development, the production of secondary metabolites, tolerance to different types of stress, hormonal balance and photosynthetic efficiency, among others (Behbahani *et al*., 2020). There are reports indicating that high concentrations of Se can cause phytotoxicity in different plant species (Garza-García *et al*., 2023).

Therefore, the effect of Se on plants depends on the species, the stage of development, and the source of selenium (León-Morales *et al*., 2019). This study aimed to induce callus formation in different explants of *C. roseus* through the combination of growth regulators and to evaluate the effect of two inorganic sources of Se on callus growth in order to identify the best range of concentrations.

This work was carried out at the facilities of the Plant Biotechnology Unit, Zapopan headquarters, of the Center for Research and Assistance in Technology and Design of the State of Jalisco (CIATEJ, for its acronym in Spanish). The seeds of *C. roseus*, Mediterranean Burgundy Halo variety (BallSeed, USA), were superficially disinfested with commercial 3% sodium hypochlorite (NaClO) for 8 and 12 min.

Subsequently, the seeds were washed three times with sterile distilled water for 3 min. On March 15, 2022, seeds were sown in 10% MS (Murashige and Skoog, 1962) medium (with 3 g L^{-1} sucrose and 8 g L^{-1} agar) and kept incubated at 25 °C. The germination percentage was determined 10 days after the experiment was established.

Explants of hypocotyls and leaves of seedlings obtained from *in vitro* culture were placed in Petri dishes with MS medium (30 g L^{-1} sucrose, 8 g L^{-1} agar) and with treatments with growth regulators (Table 1). Five leaves and three hypocotyls were placed on each dish, with a total of 10 replications per treatment. The dishes were kept incubated at 25 °C for 40 days.

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Table 1. Treatments employed with growth regulators for callus induction.

Treatment	Growth regulators (mg L^{-1})		
	2-4-D	BA	NAA
1	3	2	2
2	0	3	2
3	0	1.5	0.5

2,4-D= 2,4-dichlorophenoxyacetic acid; BA= 6-benzylaminopurine; and NAA= 3-naphthaleneacetic acid.

To evaluate the different Se concentrations, subcultures of the calluses obtained were performed with the culture medium of treatment 1 and 1, 10, 50, or 100 mg L^{-1} of Na_2SeO_3 or Na_2SeO_4 was added. After analyzing the data obtained from the first trial, a second experiment was carried out with a narrower range of concentrations (5, 10, 15, or 20 mg L^{-1} from both sources of Se) and the control.

In both experiments, the calluses were kept incubated at 25 °C with a photoperiod of 16 h light and 8 h dark for 60 days. Callus induction was determined using photographic images, and the area of each callus was measured using ImageJ software (Version 1.53s, May 2022).

Growth was assessed with fresh callus weight on an analytical balance (BP121S, Sartorius, 120 g., Goettingen, Germany). An analysis of variance and a comparison of means were performed with Duncan's multiple range test, in Statgraphics Centurion XV (StatPoint Inc., 2007), with a significance level of $p \leq 0.05$.

Ninety-three percent germination was obtained after 10 days of sowing; germination was determined by the emergence of the radicle. With the two treatments (8 and 12 min with NaClO), there was a 95% success rate in seed disinfestation. Treatment with 8 min was used for the establishment of *in vitro* culture.

For callus induction, no significant differences were found in the type of explant used (hypocotyls or leaves) ($p= 0.3665$). The growth regulator factor was statistically significant ($p= 0.0001$) for the callus area. Treatments 1 and 3 generated callogenesis, with an average callus area of 0.72 cm^2 with treatment 1 in both explants. There was no callus formation with treatment 2.

With treatment 3, 0.66 and 0.56 cm^2 were obtained in the calluses obtained from hypocotyls and leaves, respectively. Qualitative differences were observed in the calluses of treatments 1 and 3 (Figure 1). The mixture of growth regulators used in treatment 1 stimulated the formation of white and friable calluses (Figure 1a); these characteristics of the plant material are suitable for mass plate callus culture or for the establishment of suspension cell culture.

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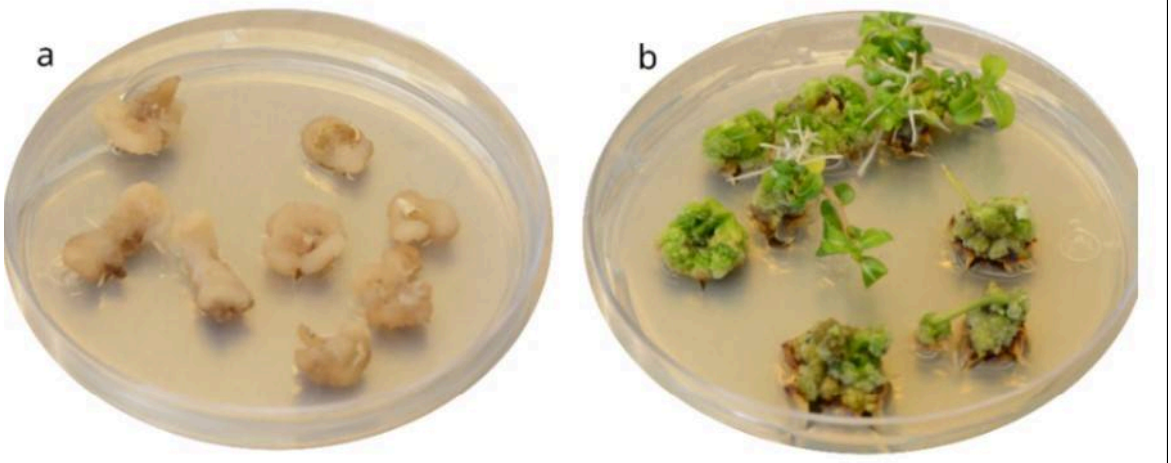
Figure 1. Callus induction in C. roseus. a) calluses obtained with treatment 1 (3 mg L^{-1} of 2,4-D, 2 mg L^{-1} of BA and 2 mg L^{-1} of NAA); and b) calluses from treatment 3 (1.5 mg L^{-1} BA and 0.5 mg L^{-1} NAA).



The results of this study are consistent with those reported by Rahman *et al*. (2019), who obtained calluses of *C. roseus* with similar characteristics in leaf explants from seedlings grown *ex vitro* in a culture medium with 3 mg L^{-1} of 2,4-D and 1 mg L^{-1} of BA. The calluses of treatment 3 (Figure 1b) had a compact and green consistency, which may be due to the production of chlorophyll; after 60 days, organogenesis processes were observed. Singh *et al*. (2011) generated calluses with similar characteristics, with 1.5 mg L^{-1} of BA and 1 mg L^{-1} of NAA.

Calluses with these properties are helpful for micropropagation due to the observed differentiation process (Das *et al*., 2020). To determine the effect of Se on callus growth, without inducing a differentiation process, calluses obtained with treatment 1 were used. The best growth of calluses was obtained with 1 and 10 mg L^{-1} of Na_2SeO_4 (0.52 and 0.53 g fresh weight, respectively) and with 1 mg L^{-1} of Na_2SeO_3 (0.5 g fresh weight); these results were superior (p= 0.0001) to the control (0.36 g).

Higher Se concentrations (50 and 100 mg L^{-1}) had a negative impact on callus growth (0.18 and 0.1 g with Na_2SeO_3 and 0.3 and 0.07 g with Na_2SeO_4, respectively) (Figure 2).

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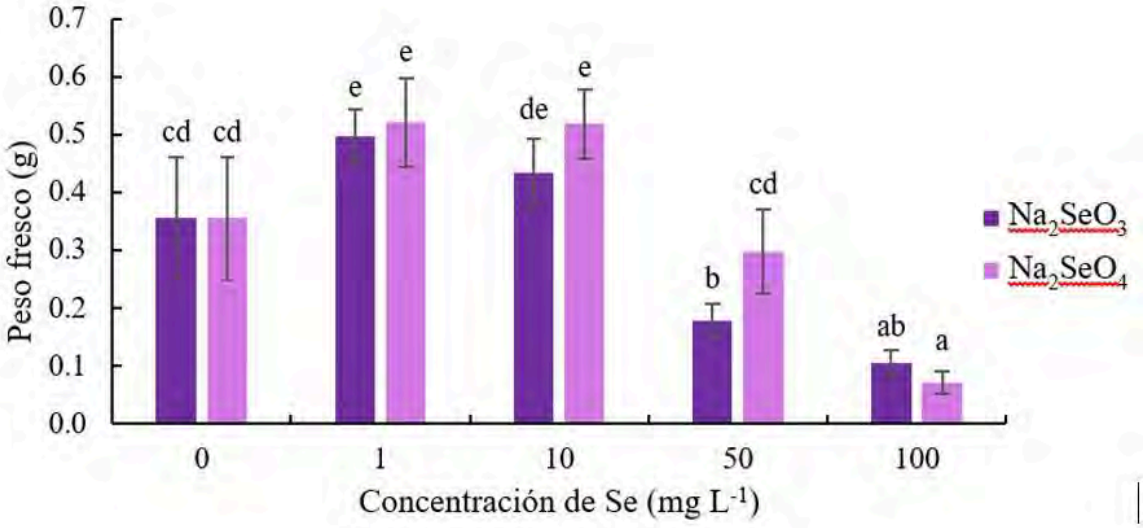
Figure 2. Fresh weight of calluses grown with two inorganic sources of selenium. The letters above the bars indicate significant differences, using Duncan's multiple range test with a significance level of $p \leq 0.05$.



Similar results were obtained in other studies with different plant species, where the application of Se positively affected plant development within a specific range of concentrations (Garza-García *et al*., 2023). Sotoodehnia-Korani *et al*. (2020) reported higher root and leaf growth of *Capsicum annuum* with 0.5 and 1 mg L^{-1} of Na_2SeO_4, while plants showed symptoms of toxicity with doses greater than 10 mg L^{-1}.

This contradicts what was observed in *C. roseus*, since 10 mg L^{-1} of Na_2SeO_4 or Na_2SeO_3 had the highest growth of calluses. In turn, Behbahani *et al*. (2020) reported larger leaf, stem, and root sizes of *Momordica charantia* (L.) grown *in vitro* when using 1 and 4 mg L^{-1} of Na_2SeO_4; however, concentrations greater than 10 mg L^{-1} decreased seedling size.

The results obtained in the first experiment allowed us to propose another trial with a smaller range of concentrations. In the second experiment, the calluses with the highest fresh weight were obtained with 5 and 10 mg L^{-1} of both Se sources (p= 0.0001). From 15 mg L^{-1} onwards, there was a decrease in the fresh weight of the calluses, although it was not statistically different from the control (p= 0.2385) (Figure 3).

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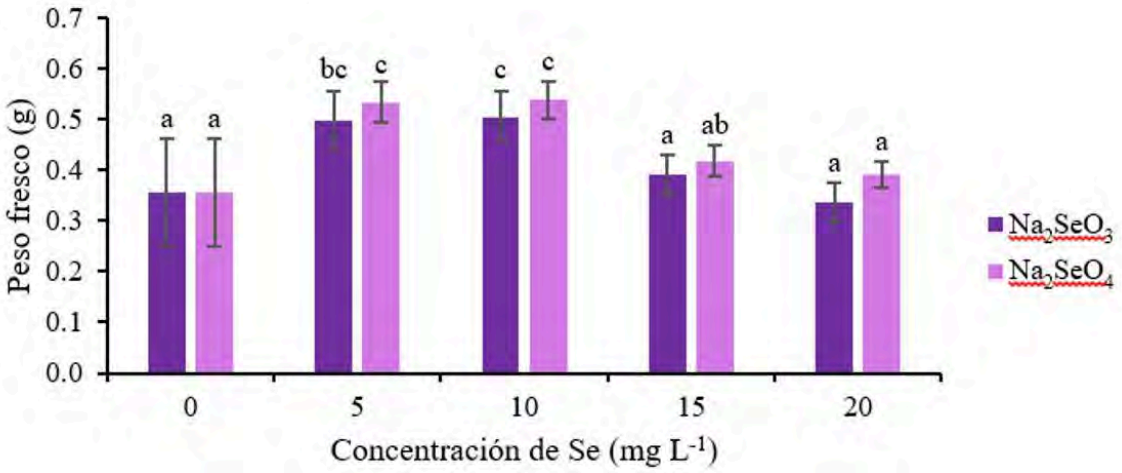
Figure 3. Fresh weight of calluses grown with two inorganic sources of selenium. The letters above the bars indicate significant differences, using Duncan's multiple range test with a significance level of $p \leq 0.05$.



In addition, differences were found between the sources of Se; the fresh weight of calluses grown with 50 mg L^{-1} of Na_2SeO_3 was statistically lower than those grown at the same concentration of Na_2SeO_4. Therefore, Na_2SeO_3 was more toxic to *C. roseus* calluses than Na_2SeO_4. Other studies have also established a comparative effect between Na_2SeO_3 and Na_2SeO_4.

In *C. annuum* plants, 5 µM of Na_2SeO_3 increased the fresh and dry weight of roots, leaves, and stem, while 5 and 10 µM of Na_2SeO_4 resulted in similar effects on growth, finding that *C. annuum* was more tolerant to Na_2SeO_4. Nevertheless, both sources of Se had a negative impact with 20 µM of Na_2SeO_3 or Na_2SeO_4 (Hernández-Hernández *et al*., 2018).

Similarly, León-Morales *et al*. (2019) found that 2.5 µM of Na_2SeO_3 or Na_2SeO_4 improved the germination percentage of *C. annuum*. Nonetheless, the growth of the aerial part was higher only in seedlings treated with Na_2SeO_3. On the other hand, in *Raphanus sativus* (L.), there were no significant differences in germination or seedling growth with Na_2SeO_3 or Na_2SeO_4. The effect of Se on plants depends on the source, concentration, species, and cell type.

Conclusions

Treatment with 2 mg L^{-1} of 6-benzylaminopurine, 2 mg L^{-1} of 3-naphthaleneacetic acid, and 3 mg L^{-1} of 2,4-dichlorophenoxyacetic acid favored the formation of friable calluses. Application of Na_2SeO_3 or Na_2SeO_4 (1, 5, or 10 mg L^{-1}) to the culture medium had a positive effect on callus size and weight, whereas a dose greater than 20 mg L^{-1} of both sources of Se negatively affected callus growth.

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