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Effect of Nitrogen on *in Vitro* Propagation of Endangered Medicinal Plant: *Swertia Chirayita* Roxb. Ex Flaming

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Abstract: The effect of nitrogen was investigated on the organogenesis of *Swertia chirayita* (Gentianaceae) to overcome the challenges related to its cultivation. The best callogenic response was observed on root explants inoculated onto MS medium supplemented with BAP (2.0 mg/l) along with 2,4-D (0.5 mg/l) after 35 days of culture. Subsequent transfer of callus for multiplication on the same media composition under complete darkness presents the best results in terms of callus multiplication. Callogenic cultures were subculture onto modified MS medium supplemented with inorganic nitrogen sources, i.e., NH_4NO_3 (14-56N/l), KNO_3 (100-400N/l) with BAP (3.0 mg/l) were observed. Organogenic response (52%) was observed after 8-12 weeks of culturing. The maximum number of the shoot was recorded on MS medium with NH_4NO_3 (28 N/l), KNO_3 (300N/l) with BAP (3.0 mg/l). Moreover, 90% of them were able to regrow when sub-cultured on the same media. Sixteen weeks old multiple shoots were subcultured on MS medium supplemented with different auxins. IAA was proved to be the best hormone rooting purpose. However, the best rooting response regarding the number of roots and an average length of roots was obtained at IAA (1.0 mg/l). Survival of 90% was achieved when rooted plantlets were successfully established in substrate containing sand, vermicompost, and garden soil in equal proportion for hardening and acclimatized.

Keywords: *Swertia chirayita*; *in vitro* plant regeneration; root explants; organogenesis; plant growth regulators.

1. Introduction

The use of traditional medicines in controlling and treating various diseases has gained a lot of popularity worldwide. Numerous traditional literature on medicinal plants' properties and use in various diseases is available in various cultures and parts of the world [1,2]. There is a growing awareness among people about the use of herbal medicines because they are safe and have no side effects [3]. Yet, the quest for the effectiveness and safety of these herbal medicines is in its infancy. Researchers are investigating the efficacy and safety of traditional plant-derived medications. *Swertia chirayita* is one such medicinal plant found in the Himalaya region at an altitude of 1200 to 2100 m from Kashmir to Bhutan and used to treat various human diseases [4]. It belongs to the family Gentianaceae and genus *Swertia*, which includes the annual and perennial herb. *Swertia chirayita* is one of the important medicinal plants among the other 40 other species of Swertia found in India. Continued harvest and overexploitation of

this plant from the natural habitat have brought it to the brink of extinction [5]. The plant is already facing issues related to seed viability and germination. The premature harvest of the plant before the seed matures limits its natural regeneration. The vegetative propagation is also not supported, and hence an alternative method for propagation is soughed to save it from extinction. *In vitro* propagation of *Swertia chirayita* is very promising and beset with many limitations [4]. Only a few reports are available on micropropagation with nodal, shoot tip explant, and lab-grown seedlings [4,6–9]. Chaudhuri *et al.* [7,8] and Wang *et al.* [10] also reported the direct shoot regeneration from leaf explant. Wawrosch *et al.* and Kshirsagar *et al.* [11,12] reported using root explants from the *in vitro* grown seedlings for shoot induction *in vitro*. Besides having some work done on *in vitro* propagation of *Swertia chirayita* there is still a dearth of an effective protocol for enhanced propagation of this plant. In the present research, the effect of nitrogen was optimized to develop a cost-effective *in vitro* regeneration protocol for *Swertia chirayita*.

2. Materials and Methods

2.1. Plant materials.

The mother plant grown in the Arboretum of G. B. Pant Institute of Himalayan Environment and Development, Sikkim Unit, Gangtok, was used to collect roots as explants. All the mother plants from which explants were collected were healthy and young. The root segments were first rinsed with tap water with tween 20, then surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for three minutes. Then, the explants were further washed with sterilized with double distilled water five times in laminar airflow to remove traces of $HgCl_2$.

2.2. Medium and culture conditions.

After surface sterilization, the root explants were maintained at basal MS medium supplemented with different concentrations of 2,4-D (0.5 - 1.0 mg/l) and BAP (0.5-5.0 mg/l)alone as well as combinations. The callus cultures were kept in the dark while the shoot cultures were kept at 25±1°C for 16 hrs in light and 8 hrs in the dark.

2.3. In vitro shoot induction.

MS medium supplemented with 2% sucrose, solidified with 0.7% agar (w/v), and different concentrations of NH_4NO_3 (14-56N/l) and KNO_3 (100-400N/l) with BAP (3.0 mg/l) was used for the induction of shoots from the callus cultures. As mentioned above, the cultures were incubated in light, and the data was recorded at regular intervals for four weeks.

2.4. In vitro shoot multiplication

After optimizing physicochemical conditions for the induction of shoots from callus culture, the shoots were transferred onto the fresh medium of similar composition for multiplication. Further, the optimization of medium and culture conditions was made to enhance the shoot multiplication. The effect of different concentrations of growth regulators *viz*. BAP (0.5 – 5 mg/l BAP) and Kn (0.5 to 3 mg/l) alone and BAP (3, 5 mg/l) in combination with α-NAA (1-2 mg/l) and IAA (0.5-5 mg/l) were used to optimize the results.

2.5. Rooting of in vitro raised shoot.

In vitro shoots were transferred to the rooting medium. The basal MS medium in combination with different auxins *viz.* α-NAA, IAA and IBA was used in the liquid and semisolid form. The different concentrations of auxins were tested to optimize the rooting response of *in vitro* raised shoots. The data was recorded after 4 and 8 weeks of culturing. The number of roots and the average length of the roots were recorded.

2.6. Hardening and acclimatization.

Macronutrients of MS medium (without growth regulators and carbon sources) were used. The plants were kept in the jam bottle containing a sterilized hardening medium.

2.7. Statistical analysis.

Experiments were conducted in a randomized block design (RBD), and in 10 replicates.

3. Results and Discussion

3.1. Initiation of callus culture:

The *in vitro* callus cultures were produced from root explants cultured on MS medium supplemented with different plant growth regulators viz concentrations. BAP (0.5-5 mg/l) and 2,4-D (0.5 - 1.0 mg/l) in combinations (Graph 1). The combinations of auxins and cytokinin are reported to enhance the induction of callus from root explants in many other dicot species [13]. In order to make the protocol reproducible and cost-effective fewer numbers and concentrations of the hormones were used. However, the callus induction was also reported on ½X MS medium containing BA, Kn (2.22–4.44 µM), α-NAA (2.69–5.37 µM), and 2.26 µM 2,4-D by Chaudhuri *et al.* [14]. Kumar *et al.* [15] reported the callusing from leaf explants of *Swertia chirayita* upon culturing on 15.0 µM 2,4-D within 60 days. Studies made by other researchers were referred to make a reach to the final optimal concentration of both the hormones for callus induction [16–18]. The best results were obtained on 2.0 mg/l BAP in combination with 0.5 mg/l 2,4-D. The maximum amount of the callus (1090 mg) was obtained at the optimal concentration and further subjected to the callus multiplication (Graph 2, Figure 1). The multiplied callus cultures were transferred on a cytokinin medium for subsequent organogenesis from callus culture. Balaraju *et al.* [19] has also reported the somatic embryogenesis from leaves and root explants of *in vitro* grown young seedlings of *Swertia chirata*. He reported that the 2,4-D (1.0 or 1.5 mg L^{-1}) alone was good for the initiation of embryogenic tissue

3.2. In vitro shoot induction.

The callus cultures were transferred on to MS medium supplemented with 3% sucrose, 0.7% agar (w/v) and different concentrations of NH_4NO_3 (14-56N/l) and KNO_3 (100-400N/l) with BAP (3.0 mg/l) (Graph 3, Graph 4). Induction of shoots from the callus cultures was observed in four weeks of culturing. The maximum number of shoots (52%) were obtained on medium with NH_4NO_3 (28 N/l), KNO_3 (300 N/l), and BAP (3.0 mg/l). Further, the maximum number of shoots (32 shoots per callus) and length (3.6 cm mean length) were also found on

the same medium (Graph 5). The elongation of shoots was also reported on MS medium with GA_3 (0.1 mg/l) [20]. Kumar *et al.* [15] reported the shoot organogenesis from callus culture on MS medium supplemented with BAP (10.0 µM) with GA_3 (5.0 µM). They also reported that BAP (5.0 µM) was also good for the induction of shoots from the callus cultures. There was a marked difference in the morphology of shoots reported when cultured on BAP alone and in BAP and GA_3 containing medium. Wawrosch *et al.* [11] reported the adventitious shoot regeneration in *Swertia chirata* upon culturing on 3 µM 6-benzylaminopurine (BAP) within 3 weeks of culturing. In another study, the shoot induction was reported on 1/2x MS medium supplemented with 0.44 µM 6-benzylaminopurine and 4.65 µM 6-furfurylaminopurine [8]. In this study, it is also found that the MS medium supplemented with 10 mM KNO_3 and 75 mg l^{-1} of casein hydrolysate produced a maximum number of shoots. In one study, the shoot tip explants from the *in vitro* grown seedlings were used to induce multiple shoots from the explants. The shoot tip explants were reported to give a maximum response on MS medium with BAP at 1.0 mg/l and KN, 0.1 mg/l [20].

3.3. In vitro shoot multiplication.

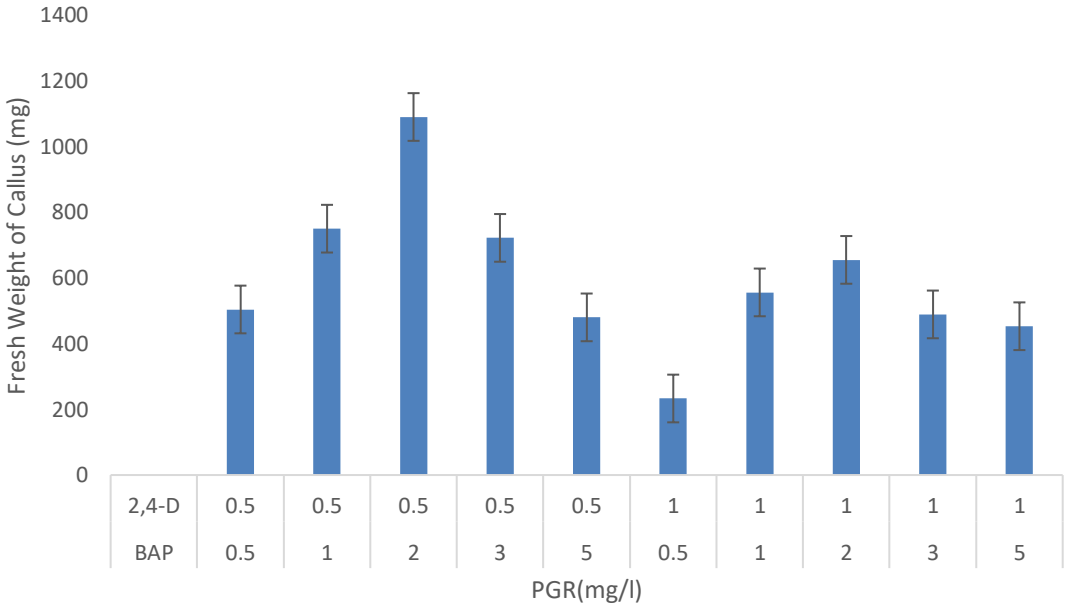
The shoots obtain from callus culture were transferred to a fresh medium with different concentrations of cytokinin and auxins (Graph 6). BAP alone (2 mg/l) and in combination with Kn(1, 3 mg/l) provided the best result (Figure 2). Around 90% of the shoots were found successfully cultured and proliferated upon sub-culture on the same media. The addition of reduced nitrogen in the medium is reported to increase the length of shoots and the number of leaves in potato cultivars [21,22]. Ammonium nitrate concentrations 3.4–10 mM and 3.4–15 mM were also reported to develop somatic embryos in black spruce and red spruce, respectively [23]. Nitrogen plays a crucial role in plant growth and differentiation. Both its amount and form have a significant effect on cell growth and differentiation. However, MS medium has a high inorganic nitrogen concentration (60 mM), supporting most plant species. Still, the standardization of the appropriate ratio of nitrate to ammonium is essential for optimal plant growth [24,25]. In the present investigation, the addition of ammonium nitrate and potassium nitrate was also found beneficial for the induction as well as multiplication of shoots.

3.4.Rooting of in vitro raised shoots.

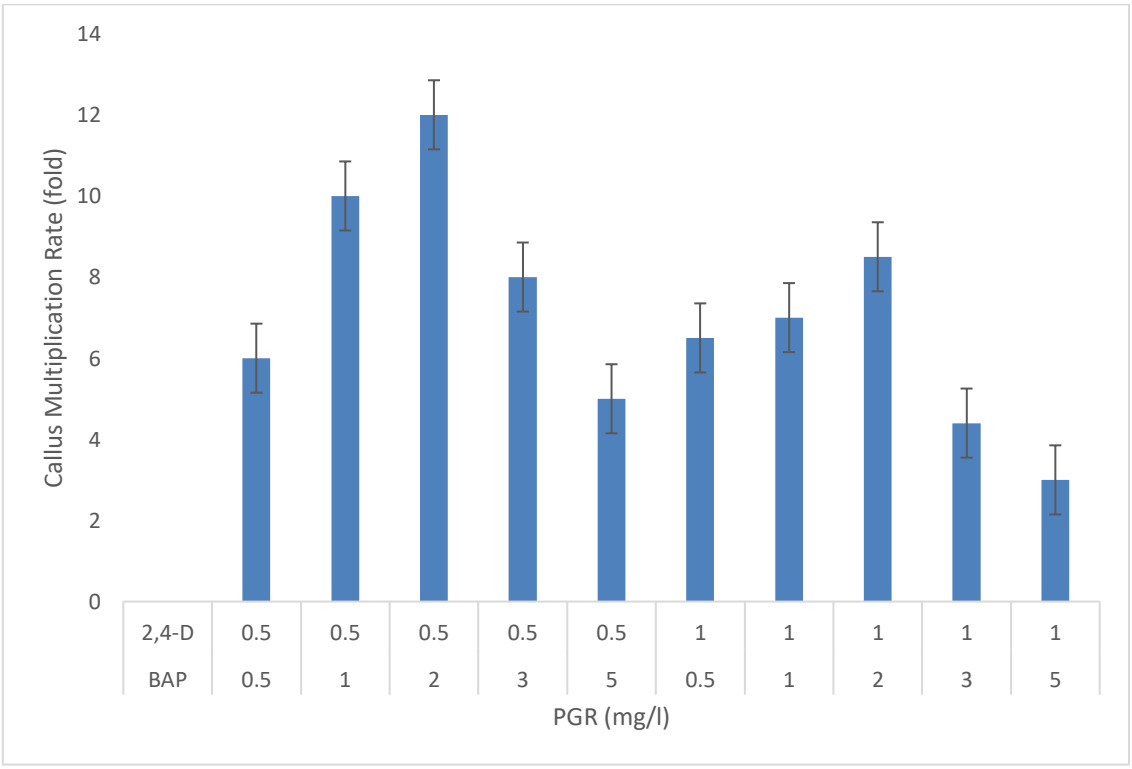
For the rooting response, multiplied shoots were transferred on MS medium with different concentrations of auxins (NAA, IAA, and IBA). Different concentrations of NAA (0.1-2.0 mg/l) was found non-significant for root induction. IBA at different concentrations (0.1-2.0 mg/l) gave the rooting response, but the maximum rooting was found on medium supplemented with IAA (1.0 mg/l) (Graph 7, Figure 2). Balaraju *et al.* [20] reported the rooting of *in vitro* raised shoots of *Swertia chirayita* on MS medium containing α-NAA, 0.1 mg/l within four weeks of culturing. In another report, the MS medium supplemented with IBA (5.0 µM) was reported to produce 50 roots per microshoot [15]in 45 days of culture. Kshirsagar *et al.* [12] reported the best rooting of *in vitro* raised shoots of another species (*Swertia lawii* Burkill) on 1/2 MS medium containing IAA (3.0 mg l^{-1}). In a similar report on *in vitro* propagation of *Swertia chirayita*, ½ MS strength supplemented with 400 mg/L activated charcoal (AC) and 0.1 mg/L α-NAA showed 80.30% root induction from *in vitro* grown shoots [26].

3.5. Hardening and acclimatization.

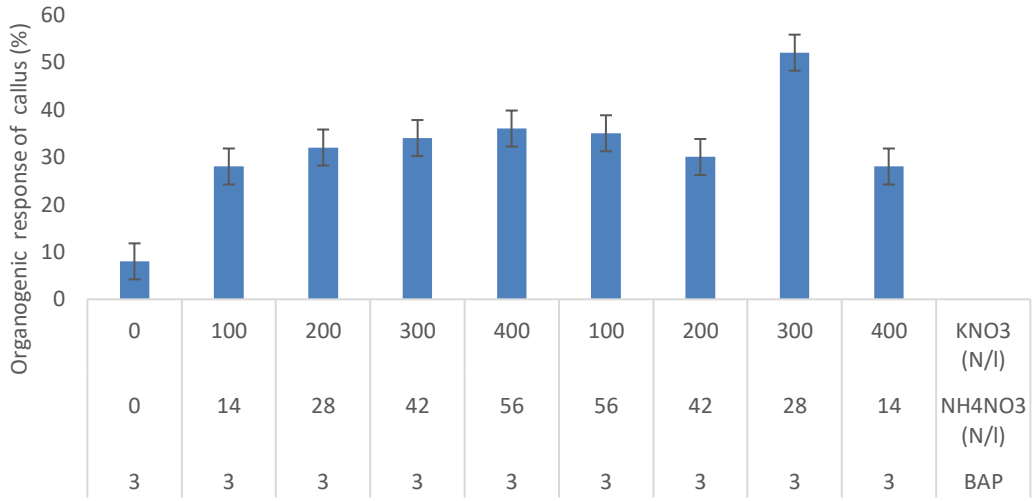
The rooted shoots were transferred on a hardening medium containing sand, vermicompost, and garden soil in equal proportion for hardening, and acclimatized 90% of the transferred plants were successfully established in the substrate (Figure 3). The hardening of well-rooted plants of *Swertia chirayita* was reported on the mixture of soil, sand, and FYM (2:1:1) under partial shade (75% agro shade net) [15]. An average 94 % success rate was reported in the *in vitro* hardening of *Swertia chirayita* in the growth room and by *ex vitro* hardening in greenhouse conditions[19,27].



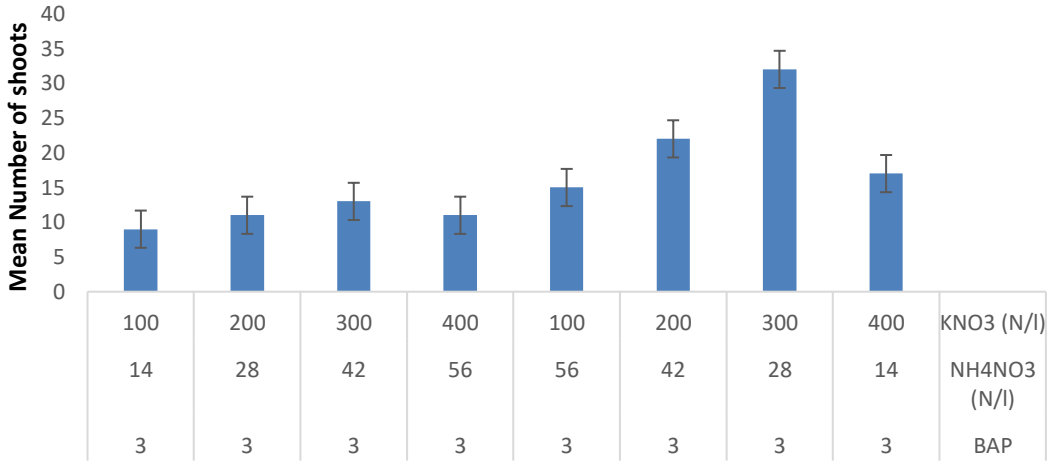
Graph 1. Effect of BAP and 2,4-D on callogenesis (fresh weight is measured in mg).



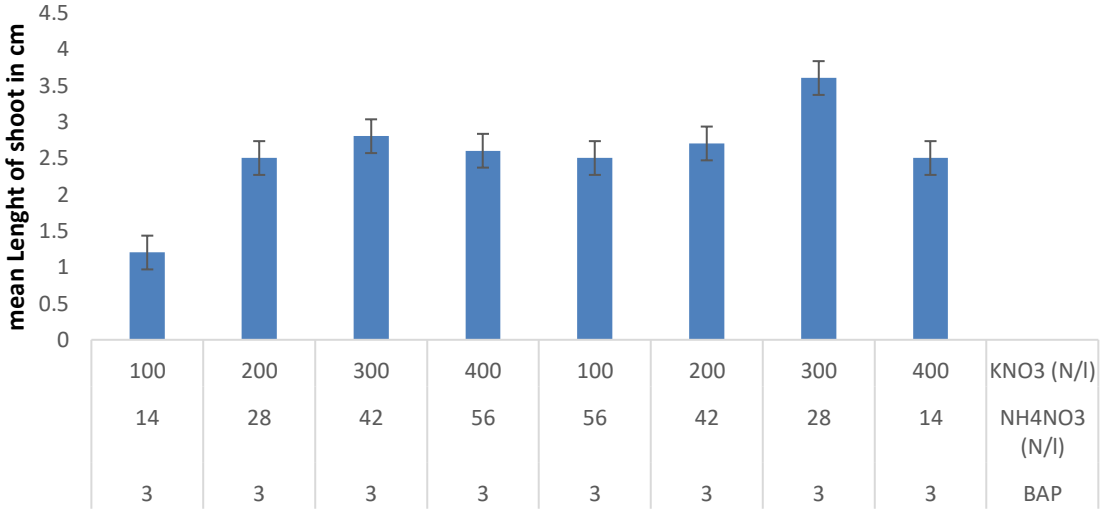
Graph 2. Effect of BAP and 2,4-D on callogenesis (Callus multiplication rate is measured in folds).



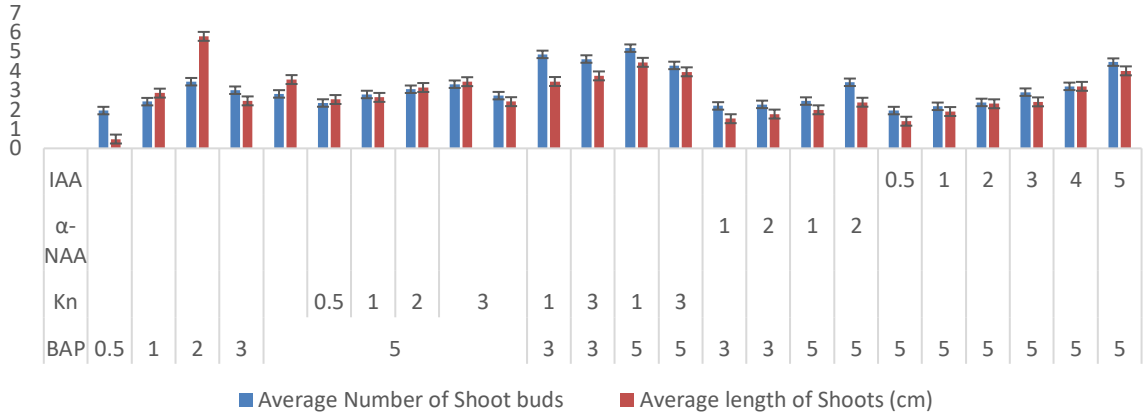
Graph 3. Effect of different concentrations of NH_4NO_3 and KNO_3 on organogenesis of callus cultures.



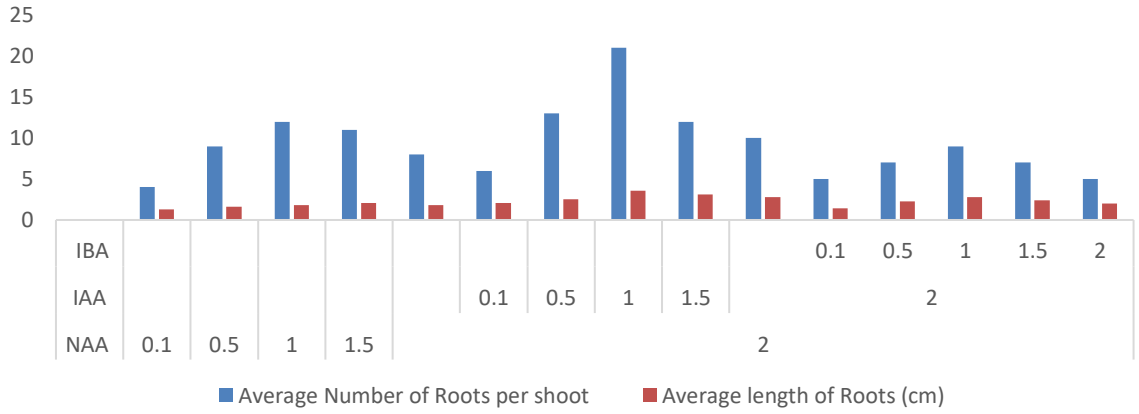
Graph 4. Effect of different concentrations of NH_4NO_3 and KNO_3 on the mean number of shoots per callus clump.



Graph 5. Effect of different concentrations of NH_4NO_3 and KNO_3 on mean length of shoots.



Graph 6. Effect of PGRs on shoot multiplication.



Graph 7. Effect of Auxins on rooting of *in vitro* shoots.

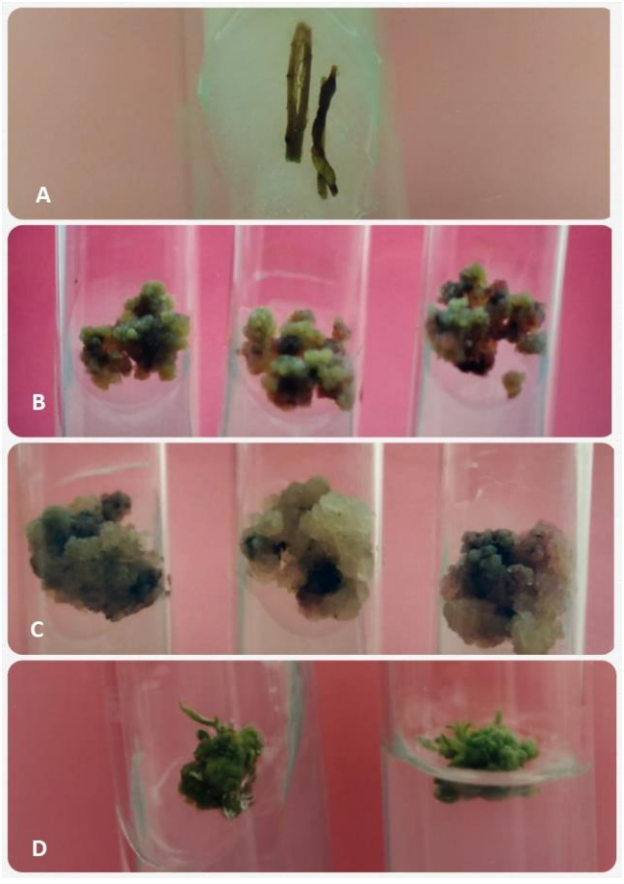


Figure 1. Initiation multiplication and organogenesis in callus culture from root explant of *Swertia chirayita*.

Figure 2. Multiplication and rooting of *in vitro* raised shoot of *Swertia chirayita*.

Figure 3. Hardening of *in vitro* rooted plantlets of *Swertia chirayita*.

4. Conclusions

This study indicates the varied responses to the different forms of inorganic nitrogen provided, representing the previously reported importance of this nutrient in determining plant morphology and morphogenesis *in vitro*.

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Conflicts of Interest

The authors declare no conflict of interest.

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