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"*In Vitro* Propagation of *Withania Somnifera*(L.) DunalA Valuable Medicinal Plant"

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Abstract

Withania somnifera (L.) Dunal commonly known as Ashwagandha, isan important medicinal herb belonging to the solanaceae family. It is rich source of withanolide A, withanolide D, withaferin A, and withanone. Presence of various pharmacological properties, the plant has huge demand in pharmaceutical industry due to its pharmacological properties. However, there is large gap between demand and supply, thus, it is necessary to develop *in vitro* propagation techniques for this medicinally valuable plant. In the present research *in vitro* propagation of, *Withania somnifera*, direct regeneration of nodal explants and their multiplication have been optimized using cytokinin BAP and Kin (2.0 µm/l). MS media with nodal explants supplemented with BAP (2.0µm /l) produced maximum average number of shoots (5±0.53) and average shoot length was found to be 4.8±0.28 cm. Shoot proliferation was observed on BAP(4.0µM)+ Kinetin (4.0µM) with maximum number of shoots 8.03 ± 0.13. Elongated shoots then sub cultured for root induction on MS media supplemented with IBA, IAA and NAA (2.0-8.0µm/l) Maximum average number (6± 0.10) and average length 7.6±0.18cm of roots were observed on MS media supplemented with IAA (2.0µm/l).The rooted plantlets when transferred to poly bags in the greenhouse showed 85% survival frequency. This protocol useful for production of large quantity withaferin and conservation of this valuable medicinal plant

Keywords:*Withania somnifera*, micro propagation, nodal explant, BAP, KIN, IBA.

Introduction

Withania somnifera, also known as Ashwagandha or Indian winter cherry, an evergreen woody shrub of the member of Solanaceae family. Itis a traditional Ayurvedic herb known for its adaptogenic properties, which help strengthen the nervous system. It has long been valued as a "rasayana" which promoting overall health and resilience to stress. (Połumackanycz et al., 2020).

Ashwagandha has been tremendously used as astringent, anti-carbuncle, anti-stress, tonic, narcotic, diuretic and against worms, piles, goitre, leucoderma, nervous breakdown, insomnia, constipation, loss of memory, heart disease, atherosclerosis, Aids and aging because of its strong anti-oxidative property. The plant is a rich source of pharmacologically active withanolides including withaferin A, known as anticancer molecule that are prospective high-value drug candidates.(Hahm et al., 2013). Leaves of plant used to treat inflammation of tubercular glands and that of its roots used for curing skin diseases, bronchitis, ulcer and dyspepsia and eye diseases. The fruits and seeds of Ashwagandha are diuretic in nature. The leaves are reported to contain anthelmintic and febrifuge properties. An infusion of the bark is used for asthma (Basiri et al., 2022)

The growing demand for bioactive withanolides has led to overexploitation of wild sources, endangering their natural populations. The pharmaceutical industry typically relies on field-grown plants, often uprooting entire plants to meet demand. However, challenges such as long growth periods, slow maturation, and low concentrations of withanolides make extraction difficult. (Mir and Khazir 2014).*W. somnifera* is traditionally propagated from seeds, but seed viability is short, and germination rates are low. Dependence on wild populations for *Withania somnifera* can be reduced by encouraging its cultivation. However, conventional methods cannot meet the demand. *In vitro* regeneration offers a scalable alternative for large-scale production, making the plant more accessible and affordable. (Autade et al., 2016).

Recent studies have explored various in vitro techniques for rapid propagation of *W. somnifera*, revealing that different culture conditions and plant growth regulators (PGRs) affect the plant's response. (Shasmita et al., 2018). BAP is commonly used for shoot proliferation, while IBA aids rooting, though other cytokinin's and auxins have also been tested. (Saema et al 2015). Prolonged use of cytokinin-rich media, however, can lead to issues like hyperhydricity and genetic instability, which are undesirable for herbal applications. (Bradaï et al., 2016).

Given the application of *W. somnifera* in the pharmaceutical industry and various preclinical studies, genetic instability and somoclonal, phytochemical variation is undesirable traits. Therefore, this investigation focused to developed an optimized protocol for *in vitro* propagation of *W. somnifera* by modifying key factors and implementing effective subculture strategies to ensure genetic fidelity,large scale production for stable *in vitro* propagation of *W. somnifera*.

Materials and Methods

Plant material and sterilization procedure.

Mother plant was collected from wild populations in Beed bypass road, Chh, Sambhajinagar Maharashtra, India(lat N, long 073.49580o E, alt 3,878 m). The specimen was identified in Department of Botany Dr. Babasaheb Ambedkar Marathwada University.Healthy node explants were separated from the mother plant and washed under running tap water (Figure 1).

Surface sterilization of nodal explants

Nodal explants excised from mother plant were used source of explants for the induction of shoot cultures.(Figure 2) The explants were washed thoroughly under running tap water for 15 min and soaked in 1 % Bavistin for 10 min, followed by four time washing sterile distilled water for 30 min to remove the traces of Bavistin. Further they were treated with Bavistin (1 % w/v) for 10 min and sterile water was given for 10 minutes followed by treatment with 0.1 % (w/v) $HgCl_2$ for 8 min. the explants were rinsed four times in sterilized distilled water for 20 minutes each wash for 5 minutes. After surface sterilization all explants were inoculated on MS media supplemented with different concentrations and combinations of BAP + Kinetin (0.5-2μM), for shoot induction. Induced shoots transferred to fresh medium every 4 weeks. The total number of shoots, and their length, were recorded after 4 weeks of fifth subculture, using at least 10 explants per medium Induced shoots were transferred on shoot proliferation MS medium of BAP(4.0μM). + Kinetin (4.0μM). proliferating shoots, excised and transferred on root induction Ms medium supplemented IBA(0.1-0.5 μM)+ Kinetin (1 μM kin).

Culture conditions.

The basal MS mediawas used with different concentration of growthregulators (BAP, IBA, and kinetin). All culture media were agitated with 0.8% agar and3% sucrose. The pH of media was adjusted to 5.8 before addition of agar. (Figure 3). These media were autoclaved at 121 °C for15

min at 15 psi. Cultures were maintained in a culture room incubated with a 16-h lightand 8 hour dark cycle and temperaturemaintained at 24± 1 °C with 65% humidity.

Shoot bud initiation. Full strength MS media with different concentration of BAP and kinetin (0.5, l.0, 1.5, 2.0 μM), were used for shoot buds' initiation. The percentage of shoot induction, time taken for bud initiation and the growth state of the buds were measured after four weeks of culturing.

Shoot proliferation. Induced shoots were excised from cultured plant and transferred into MS media fortified withBAP(4.0μM) + Kinetin (4.0μM), to enhance the frequency of shoot multiplication.

Root initiation. To optimize root induction media, full-strength MS media was supplemented with different combination and concentrations of IBA, IAA and NAA (2.0-8.0μM).The duration to root initiation was observed and recorded after every two days. Data on average root numbers and length were recorded after 28 days of culturing.

Acclimatization and Ex Vitro Conditions

The rooted plantlets were subjected to hardening and ex vitro transfer. The rooted plantlets attaining height 5cm were transferred to hardening portrays containing autoclaved perlite:coco peat: vermiculite (2:1:1; volume). The hardening media was pre-soaked with either 1/2 MS was devoid of vitamins and sucrose. The pre-soaked substrate was subjected to autoclaving prior to plantlet transfer. The portrays were covered with transparent polythen bags and shifted to cool-white fluorescent lights with controlled photoperiod (16/8 h) and temperature (25^0 C) conditions. After two weeks, the polythene bags were loosened gradually, and finally, removed completely.At regular intervals, the growing plantlets were then moistened with sterile distilled watercontaining 0.1% bavistin (w/v) fungicide. Hardened plantlets were transferred toex vitro glasshouse conditions at the 2 weeks of acclimation.

Statistical Analysis

All experiments were arranged in a completely randomized design, and the data weresubjected to analysis of variance using SAS software .Comparisons of means were tested using the least significant difference (LSD) test,at a 0.05 level of probability (p).

Results

Shoot bud induction

Duringculture establishment of explants it has been shown that nodal explants cultured on MS media devoid of growth hormone (Cytokinin) failed to induce the shoot induction of node explants.(Figure 2)The nodal explants were derived from field grown plant of *W. somnifera* and inoculated in MS medium supplemented with different concentration of BAP and Kinetin (0.5, l.0, 1.5, 2.0 μM), The emergence of shoot buds from nodal explants was enhanced with the increased of BAP and Kinetin to the MS medium.Shoot buds induction was observed after 12 to 14 days of culturing.The rapid shoot bud initiations were observed on media MS media supplemented with cytokinin in combination of BAP and Kin (2.0 μm/l) . Increased concentration of BAP and Kinetin leads in rapid shoot buds induction. The findings revealed BAP and kinetin in combination had a marked influence on the frequency of earlier shoot induction. Similarly, BAP and kinetin in low concentration, the induction rate was decreased and the lateral buds formation observed delayed. Subsequently new buds were relatively weak. ANOVA revealed that shoot bud induction was highly significant in BAP and Kin (2.0 μm/l) among the treatments (Figure 4)

Sr. No	**MS medium + BAP+ kinetin μM/L**	**Avg. shoot number (mean±SE)**	**Avg. shoot length (cm) (mean±SE)**
1	MS medium + 0.0 (control)	-	-
2	MS medium + BAP(0.5μM) + Kinetin (0.5μM)	1 ± 0.28	1 ± 0.26
3	MS medium + BAP(1.0μM) + Kinetin (1.0μM)	2 ± 0.16	1.4±0.24
4	MS medium + BAP(1.5μM) + Kinetin (1.5μM)	3.7 ± 0.46	2.4±0.37
5	MS medium + BAP(2.0μM) + Kinetin (2.0μM)	5±0.53	4.8±0.28

Table 1: Effect of BAP and Kinetin on shoot initiation from nodal explant

Shoot bud proliferation.

The induced shoots developed from the nodal explants were transferred on shoot proliferation medium. In the current findings, the effect of cytokinin's in combination (BAP and KIN) was observed on the shoot proliferation and shoot elongation of *W. somnifera*. Various concentration of cytokinin's revealed a significant effect on the shoot proliferation. Among all the various

combination cytokinin's tested, MS media supplemented with BAP(4.0µM) + Kinetin (4.0µM) was proved to best for shoot proliferation (Table 2). The maximum number of shoots per explant (8.03 ± 0.13) and the maximum shoot length 6 ± 0.26 (Fig 5-8) were observed in MS medium supplemented with BAP (4.0µM) + Kinetin (4.0µM) compared with the other treatments (Fig. 9). Significant variation was observed in shoot multiplication rate and numbers of shoots among the given concentration of cytokinin's. Enhanced in the concentration of BAP and kinetin till 10 µM, later which a resulted in decline in the number of shoots produced per explant. However, at lower BAP and kinetin concentrations (4 µM), shoots continued, lush greenish, healthy with no marks of vitrification, however higher concentrations (10.0µM) resulted in vitrification. KIN in combination with BAP(4 µM), was revealed to be most effective on shoot proliferation (Figure 10).

Sr. No	**MS medium + BAP+ kinetin µM/L**	**Avg. shoot number (Mean±SE)**	**Avg. shoot length (cm) (Mean±SE)**
2	MS medium + BAP(4.0µM) + Kinetin (4.0µM)	8.03 ± 0.13	6 ± 0.26
3	MS medium + BAP(6.0µM) + Kinetin (6.0µM)	3 ± 0.32	4.2 ±0.18
4	MS medium + BAP(8.0µM) + Kinetin (8.0µM)	2 ± 0.19	2.1±0.37
5	MSmedium + BAP(10.0µM) + Kinetin (10.0µM)	1.8±0.61	2±0.33

Table 2: Effect of BAP and Kinetin on shoot proliferation from nodal explant.

***In vitro* rootingof *W. somnifera* shoots**

W. somnifera shoots elongated shoots were subculture in MS medium containing of different types and concentrations of auxins (NAA IBA, and IBA) were used for rooting experiments. Root initiation was observed within 10-12 days of shoot subculture in root MS medium supplemented with IAA (2.0 µM). Different auxins used for the rooting of *Withania* shoots shown a significant difference in the root formation and root elongation. Among different auxins used in the present investigation, the highest root length **8**.6± 0.28 and number of roots were recorded 18± 0.16 in IAA (2.0 µM) (Table 3). However, a maximum number of roots (12± 0.13) were recorded and themaximum root length (6.0± 0.16) was observed in IBA (2.0 µM). Root length and number of roots significantly decreased with an increase in the concentration of both IAA and IBA. Roots induced from the shoots grown in IBA medium were thick and have several lateral branches, In the concentration used in NAA, root length 2.8± 0.15 and root number (3.7±

0.16) were observed in the concentration of NAA (2.0 µM). IAA (2.0 µM) was proven to the best-suited auxin for *in vitro* rooting and produced more roots in comparison to NAA and IBA (Figure 11).

Sr. No	Auxins (µM)	No. of roots per shoot	Root length
1	IAA 2.0	**18± 0.16**	**8.6± 0.28**
2	IAA 4.0	11± 0.22	6.7± 0.32
3	IAA 6.0	8.3± 0.25	5.8± 0.32
4	IAA 8.0	6.2± 0.37	3.7± 0.18
5	IBA 2.0	**12± 0.13**	**6.0± 0.16**
6	IBA 4.0	10± 0.20	4.0± 0.28
7	IBA 6.0	6.0± 0.28	3.0± 0.36
8	IBA 8.0	4.0± 0.13	2.0± 0.23
9	NAA2.0	**3.7± 0.16**	**2.8± 0.15**
10	NAA4.0	3.2± 0.22	2.3± 0.33
11	NAA6.0	2.3± 0.25	1.8± 0.43
12	NAA8.0	2.0± 0.37	1.5± 0.46

Table 3: Auxins (µM) Number of roots per shoot Root length (cm)

Discussion

Withania somnifera, also known as Ashwagandha, is highly valued for its healing properties, specificallyin the bioactive compounds like withanolides. (Dar et al., 2016). conventionally seed propagation has certain restriction due to poor germination rates and incompatible secondary metabolite range over batches, which are affected by environmental conditions. (Wu et al., 2020) *In vitro* propagation givesa rapid, uniform plant multiplication. The results which were observed in this study indicate that the combination of cytokinin (BAP and Kinetin) to the MS basal medium resulting in significantly effective as maximum shoot induction. It is also revealed that the in combination BAP and Kinetin used have shown green, healthy plants with vigorous growth. Our findings were similar with various reported about the effect of cytokinin's for enhancing shoot multiplication of sugarcane (Ajadi et al., 2018; Rahman et al., 2020) explants showed decline shoot induction viz., frequency of shoot regeneration (%) and number of shoots per explant with

increase in concentration of BAP and Kinetin and at higher concentrations decline in growth rate of plants is observed. These findings were similar with (Kumar et al., 2020; Azu et al., 2022). The shoot induction not observed in control where there is no presence of hormones in the basal MS media. Thus, BAP incombination with Kinetin had extensive effect on *in-vitro* shoot multiplication in *Withania somnifera*. However BAP and kinetin is the significantly used cytokinin for shoot proliferation in *W. somnifera* (Savitri et al., 2018). The role of crucial role in BAPand Kinetin is the stimulation of cell division and elongation in meristems to increase the synthesis of RNA and protein (Naz et al., 2015). It also prevents senescence (Sosnowski et al., 2019). It was also revealed that, at higher concentrations of cytokinin's, shoot number and length were significantly decreased.

This finding is similar within the earlier reports where shoot length and number reduced with increasing the concentration of BAP and KIN in *W. somnifera* (Fatima and Anis, 2012). This may be due to that the supra-optimal level of cytokinin in plants resulting as stress to the upregulation of cytokinin oxidases/dehydrogenases. (Rosa et al., 2018).Rooting and acclimation of *in vitro* plantlets are the critical steps in micro-propagation. IAA the most commonly used auxin for root initiation, the maximum response of percentage of root induction and average number of roots per shoot. (Baskaran et al., 2013) NAA and IBA induced callusing prior to rooting. The survival of regenerated plants was 85 % and they grew normally after planting into plastic pots containing a mixture of sterile soiland sand (1:1 v/v) with 15 % vermiculite followed by the maintenance in the green house (Figure 7).The auxin triggers variousgenes and enhances the enzyme binding that regulate various pathways of proteins, carbohydrates, nitrogen, and polyphenolic metabolism whichresulting to rooting in plants (Bhardwaj et al., 2018). In this present investigationemployed auxins gave variousroot induction patterns and IAA (2.0 μM) was found to be best for induction of root number.

Previous, there are many findings which shown that IAA as the most effective auxin used in the range of 2.5–24 μM for the induction of rooting in *W. somnifera.* (Autade et al., 2016). At the significant range of auxin is employed for the root induction which rely on the endogenous levels of auxin at the time of the rooting of micro shoots (Sousa et al., 2013). Decreasing in in the root length was found in the higher concentration of auxin. The auxins enhance maximum root induction when employedlower the toxic level (Yan et al., 2014). This is in accordance with the previous findings where increased in concentrations of auxin prevent rooting (Saha et al., 2016).

However, the higher concentration of NAA (5.0–10.0 μM), itindices callus formation at shoot base and rooting was entirely prevented. The survival rate of regenerated plants was 85 % and they grew normally after planting into plastic pots containing a mixture of sterile soil and sand (1:1 v/v) with 15 % vermiculite followed by the maintenance in the green house (Figure12).

Conclusion

We observed that the plant can rapidly propagated *in vitro* with the application of PGRs. Nodal explants were easily initiated in a basal MS medium supplemented with equal proportion of BAP and kinetin. Increasing the concentration, the tested PGRs enhanced the regeneration and rooting response of the plant. Future studies may focus on extraction of withanolides.The best results in terms of growth and shoot development were obtained by adding BAP and kinetin in combination to the propagation medium and IBA to the rooting medium. Results of 85% acclimatization allowed good establishment of the plant in open-field conditions. The present study has successfully demonstrated an in vitro micropropagation of rare medicinal plant *Withania somnifera*, it is therefore, revealed that in vitro micropropagation techniques would serve as a cost-effective alternative for the production and conservation of this plant. in vitro-grown W. somnifera plants can serve as an alternative source for pharmaceutical research, enhancing our understanding of secondary metabolite production and supporting further advancements in this valuable medicinal plant.

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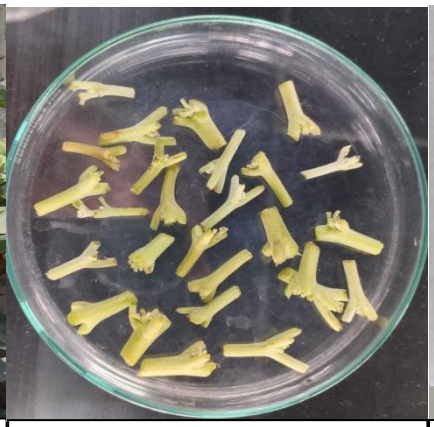

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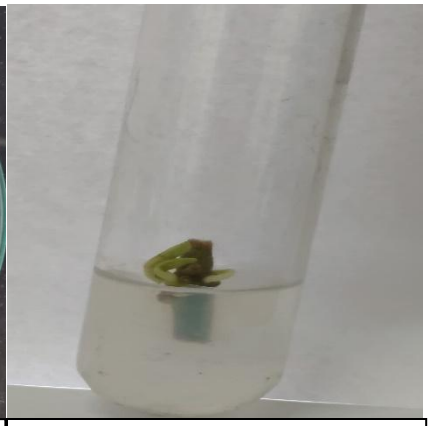

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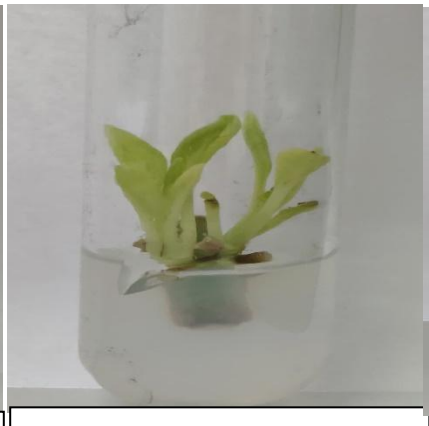

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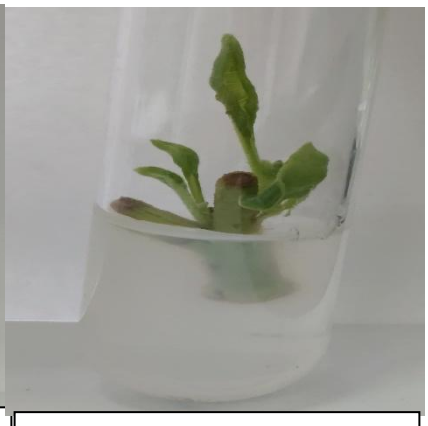

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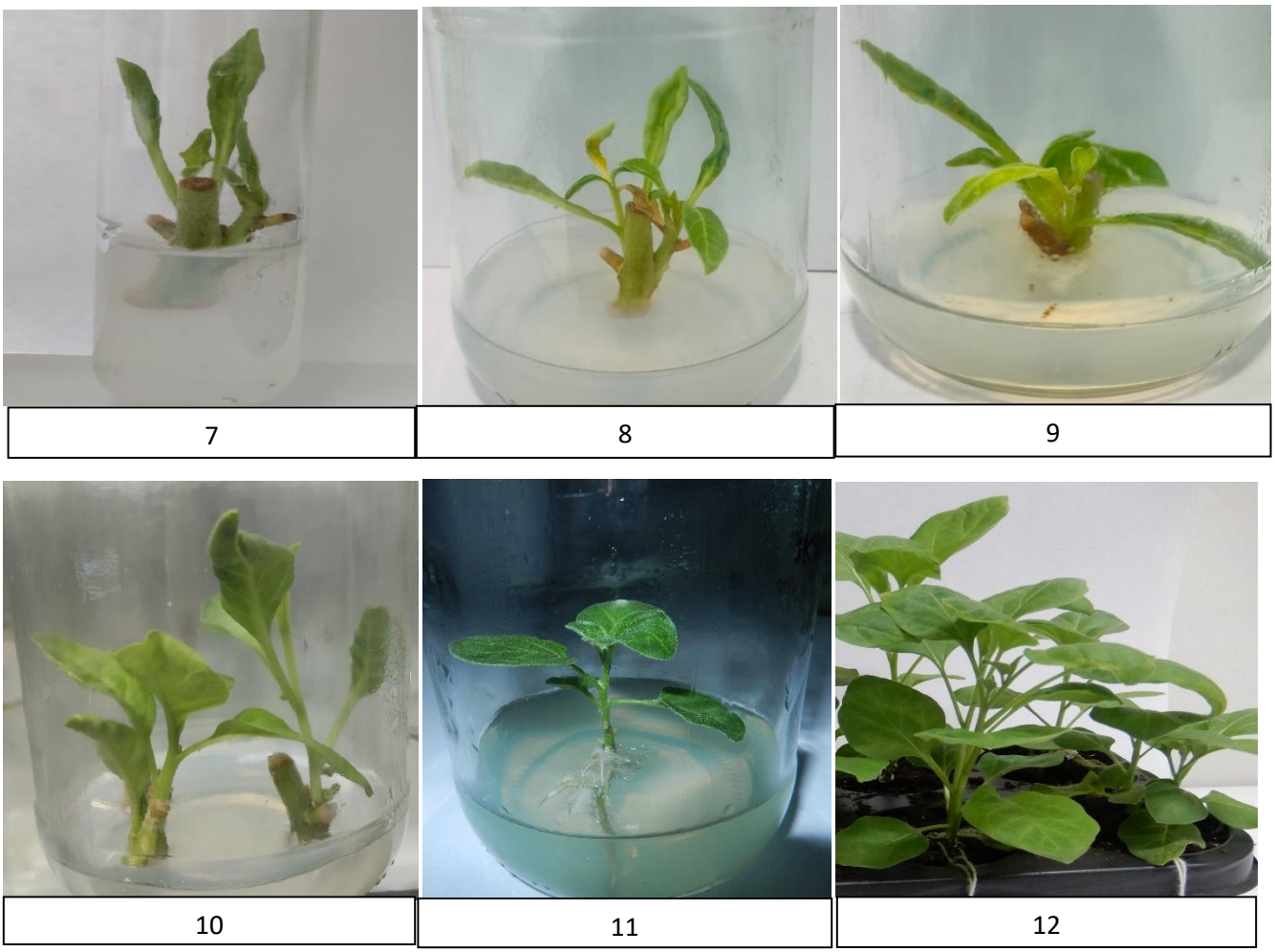

Figure: Plant regeneration of *W.somnifera* from nodal explants

1)*Withaniasomnifera* mother plant. 2) Excised nodal explants 3) Shoot initiationfrom nodal ex plant on MS medium with 2.0 µM BAP and Kinetin after 1 weeks; 4) Shoot differentiationafter 2 weeks(5) to (9) Shoot proliferationon MS medium supplemented with BAP(4.0µM) + Kinetin (4.0µM)10) Shoot elongation 11) Rooting of shoots on MS medium containing IAA 2.0µM; 12) Acclimatized plants in plastic pots in green house after six weeks.