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INFLUENCE OF SILVER NITRATE ON *IN VITRO* CALLUS INDUCTION AND INDIRECT SHOOT ORGANOGENESIS OF *SOLANUM NIGRUM* (L.) -AN IMPORTANT ANTIULCER MEDICINAL PLANT

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ABSTRACT

An efficient protocol devised for rapid callus induction and plantlet regeneration from young leaves, intermodal explants of Solanum nigrum (L.) was described. For in vitro callus induction auxins such as 2, 4-D, IAA and NAA in combination with cytokinin BAP and ethylene inhibitor silver nitrate ($AgNO_3$) were used. High frequency of creamish green, nodular callus was obtained in leaf explants cultured on MS medium supplemented with 2.0 mg/l NAA + 0.5 mg/l BAP + 0.2mg/l $AgNO_3$. The present study also describes successful plant regeneration from in vitro derived callus of young leaves. BAP, $AgNO_3$ in combination with NAA and IAA was used for regeneration of plantlets from callus culture. High frequency and maximum number of multiple shoots (15.6) were induced on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA + 0.4mg/l $AgNO_3$. All the in vitro raised shoots with a length of 3-5 cm were transferred to rooting medium supplemented with different concentrations of NAA, IBA and IAA (1.0 – 3.0 mg/l) and $AgNO_3$ (0.4mg/l). The well rooted plantlets were transferred to polybags containing soil + vermiculite in 1: 1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions for maximum survivability.

KEY WORDS

Callus induction, Plant regeneration, Leaf, Internode, Solanm nigrum (L.)

INTRODUCTION

Solanacae family comprises a number of plants widely known for the presence of variety of natural products of medicinal significance mainly steroidal lactones, glycosides, alkaloids and flavanoids. Solanum nigrum L. (Black night shade) a member of the solanacae, has a wide range of medicinal values. The herb is antiseptic, antidysentric and antidiuretic used in the treatment of cardiac, skin disease, psoriasis, herpivirus and inflammation of kidney. The fruits and leaves have been traditionally used against various nerve disorders [1]. Solanum nigrum presently grown as a homestead plant, it is often cultivated in homestead gardens as pot plants. The plant has been considered ethonobotanically important due to its use in traditional and health care system for curing severe ulcers, gastritis and stomach ache. Most prominent medicinal properties are the presence of alkaloids, solamargin and solasonine which yield solasodine as glycone has great demand in pharmaceutical industries. Solasodine has embryogenic, teratonic as well as antifungal and antiviral activities [2].

Considering the high economical and pharmacological importance of secondary metabolites, industries are deeply interested in utilizing plant tissue culture technology for large scale production of these substances. A callus culture system offers many advantages as a model system for several biological investigations. Callus cultures have been used widely in various physiological and related studies in the genus Rosa [3] and in the genus Citrus [4]. Even callus has proved better for the synthesis of alkaloids in several cases [5]. Hence present investigation was undertaken to study the callus induction, multiple shoot regeneration using young leaf, internodal explants of Solanum nigrum.

MATERIALS AND METHODS

Source of plant material

Healthy axillary bud and leaf explants of Solanum nigrum (L.) were collected from two-month-old seed germinated field grown plants growing in the Herbal garden of Dravidian University, Kuppam, Andhra Pradesh, India.

Surface sterilization

Explants were washed thoroughly under running tap water to remove traces of dust etc. followed by treatment with 10% teepol or tween -20 for 5 minutes and 0.4% bavistine (fungicide) for 10-15 minutes. Then the explants were sterilized in 70% alcohol for a minute, and finally with 0.01% mercuric chloride for1-2 minutes and washed 3-4 times with sterile double distilled water.

Culture Medium

The explants were inoculated on MS medium [6] containing 3% sucrose and gelled with 0.8% agar, supplemented with various concentrations of 2,4-D, IAA and NAA in combination with BAP and $AgNO_3$. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 minutes at 121°C and 15 lbs pressure.

Sub culturing

The cultures were maintained by regular subculture at 4 week intervals on fresh MS medium.

The frequency of callus induction was calculated by applying the following formula

$$\textbf{Callus induction frequency (\%)} = \frac{\textbf{No. of internodal explants produced calli}}{\textbf{No. of internodal explants cultured}} \times 100$$

Culture conditions

All cultures of Solanum nigrum were maintained in a culture room at temperature of 24 ± 2°C and 55-65% RH with 16 h/8 h photoperiod at a photon flux density of 3000 lux or 50-70 Em^{-2} s^{-1} provided by cool white fluorescent tubes.

Data collection and statistical analysis

Visual observations were recorded on the frequency in terms of number of cultures responding for axillary shoot proliferation, shoot development, number of shoots per explant, average length of the regenerated shoots, number of flowers and number of roots per shoot and average root length.

Despite scarcity and limitations encountered with the plant material, for most of the treatment a minimum of 10 replicates were used. All the experiments were repeated at least twice/thrice and the cultures were observed at regular intervals. The qualitative data were subjected to statistical analysis by using standard error (SE±) for shoot length, rate of shoot multiplication and then number of roots per shoot.

RESULTS AND DISCUSSION

Effect of various concentrations of $AgNO_3$ and growth hormones on callus initiation from leaf and nodal explants

For callus induction, young leaf explants were collected from the two month old field grown plants. Leaf segments (0.5-1.0 cm length) were inoculated on MS medium fortified with different concentration of $AgNO_3$ and auxins (NAA, 2, 4-D and IAA) along with 0.5 mg/l BAP shows varied callusing response. Leaf explants fail to produce the callus on media devoid of growth regulators and $AgNO_3$. But initially they were swollen slightly and gradually turned to pale yellow colour after 3 weeks of inoculation. Callus initiation has started from the cut ends of leaf explants. Initially leaf explants enlarged in size and gradually irregular cellular mass from cut edges were formed after one week of inoculation. Although the nature of the response varied with the concentration of different auxins used.

Callus induced from young leaf segments were creamish green, whitish green, dark brown to light brown, fragile to nodular callus on media supplemented with $AgNO_3$ at various combinations of growth hormones. In the $AgNO_3$ absentia, callus culturing at all hormonal concentration is resulted in very low and light yellow coloured callus formation. This indicates $AgNO_3$ had significantly provoked more shoot regenerative callus than those initiated on $AgNO_3$ free media. This might be because of suppression of callus proliferation due to over accumulation of ethylene in culture tubes. Similar enhancement was observed [7] in Brassica oleracea and [8] in buffalo grass.

Explants in the media supplemented with 0.2 mg/l and 0.4 mg/l of $AgNO_3$ at all tested concentrations of growth hormones improved the regeneration frequency, callus size and texture, whereas at lower and higher concentrations of $AgNO_3$ the regeneration frequency of callus was reduced. This might be because lower concentration of $AgNO_3$ may not be able to control detrimental effects of ethylene and higher concentration of $AgNO_3$ might have suppressed the growth rate of callus due to toxicity of metal ions.

Experimental results revealed that (2 mg/l) NAA and (0.2 mg/l) $AgNO_3$ supplemented media along with (0.5 mg/l) BAP found to be potent hormonal combination for stimulating creamish green, nodular callus induction from leaf explants. Whereas IAA (1.0 mg/l), (0.4 mg/l) $AgNO_3$ and (0.5 mg/l) BAP (Fig-1) supplemented media formed white green, compact callus. Another hormonal combination such as 2, 4-D (2.0 mg/l), $AgNO_3$ (0.4 mg/l) and BAP (0.5 mg/l) formed dark brown, compact callus. This is because 2, 4-D might have stimulated phenols, alkaloids and other chemicals, which subsequently polymerize to form brown products. This brown callus upon sub culturing did not show any further growth. 2.0 mg/l concentration of auxins (NAA, 2, 4-D and IAA) at 0.4 mg/l $AgNO_3$ and 0.5 mg/l BAP yielded green colour nodular callus with shoot tips, this upon sub culturing proliferated well. Compare to other auxins supplemented in the media, 2, 4-D had shown less callus.

Nodal explants (1.0-1.5 cm) from two month old field grown plants were cultured on MS medium supplemented with various concentrations of $AgNO_3$ and combinations of auxins (NAA, IAA and 2,4-D) and cytokinins BAP. Callusing response was varied depending on the concentration of silver nitrate and combination of growth regulators used. Callus initiated from the cut edges of the nodal explants after 6-8 days of initiation. In 2, 4-D (2.0 mg/l), $AgNO_3$ (0.2 mg/l) and BAP (0.5 mg/l) supplemented media profuse light green, compact callus was induced from nodal explants. Similar results are appeared in maize callus cultures [9].

The precise mechanism of $AgNO_3$ action on callus initiation and proliferation is not yet studied completely. However, by considering a few obtainable observations, we can assume that $AgNO_3$ might be supplying threshold level of hormones to cells for unorganized cellular growth upon continuous interaction with oozed endogenous hormones from cut ends of explants, accumulated and exogenously supplied hormones. We can also presume that silver ions (Ag^+) might have non-competitively blocked the ethylene perception by altering conformation of ETR1 (Ethylene receptor in cell walls) upon replacing Cu^+ in the cofactor [10]. Further molecular level studies have to be taken up for better understanding of $AgNO_3$ impact on callus proliferation.

Effect of $AgNO_3$ on indirect shoot regeneration from callus derived from the leaf explants of Solanum nigrum plants

Plant propagation through callus required the induction of organogenic callus. Vegetative plant parts especially leaves are desirable explants for in vitro improvement because of regeneration from these explants would preserve the genetic homozygosity of the parent genotype.

Potentiality of in vitro raised organogenic callus under the influence of silver nitrate at various concentrations of growth regulators was evaluated in terms of indirect organogenesis. Results depicted in table-16 apparently signify the positive impact of the silver nitrate on shoot regeneration frequency and shoot proliferation. Brown colour compact callus explants used for the studies did not show any growth but gradually dried. Recalcitration of brown callus can be attributed to presence of phenols, alkaloids and other phytochemicals, which might have suppressed the regenerating capacity of embryogenic callus, whereas, green nodular callus explants had shown good response for shoot regenerating cytokinin and auxin supplemented media.

Well profused callus derived from young leaf explant was sub cultured on fresh MS medium supplemented with different concentrations of $AgNO_3$, BAP and in combination with auxins NAA, IAA. After two weeks of subculture, shoot buds were emerged from leaf derived callus. The cytodifferentiation during organogenesis is always influenced by the existence of threshold concentrations of phytohormones. The addition of 2.0 mg/l BAP and 0.5 mg/l NAA along with 0.4 mg/l $AgNO_3$ (Fig-1) in the regenerating medium

gave the best overall regeneration response with maximum regeneration frequency (90%) and highest number of shoots (15.6 ± 0.29). The presence of higher cytokinins to auxins ratio is necessary for induction of indirect adventitious shoots [11], whereas at the same regeneration concentration on $AgNO_3$ free medium, embryogenic callus regenerated lesser number of shoots (5.5 ± 0.22).

This accomplished the promotive effect of $AgNO_3$ on shoot regeneration. The maximum lengths of shoots (6.6 ± 0.25cm) were inoculated in presence of $AgNO_3$ supplemented media at 2.0 mg/l BAP and 0.5 mg/l NAA. Presence of silver nitrate might have counteracted the ethylene action to enhance the shoot regeneration by perturbing the ethylene ion binding site [12]. With the stimulus of endogenous growth substance or by addition of exogenous growth regulators to the nutrient medium, cell growth and tissue differentiation are induced [13]. Our results are in accordance with previous reports of shoot regeneration in buffalo grass [8], Zea mays [15].

Effect of silver nitrate on in vitro Rooting

In vitro derived shoots at a length of 3-5cm were separated from shoot clumps and transferred to half strength MS rooting medium supplemented with different auxins such as NAA, IAA or IBA (1.0-3.0mg/l) along with $AgNO_3$ (0.4mg/l) fortified with 3% sucrose. Among the different auxins tried with half strength MS medium, IBA at (2.0mg/l) resulted in inducing maximum number of in vitro roots per shoot (39.4) with a maximum shoot length of (5.4cm), (Table-4; Fig-2).

Presence of silver nitrate in in vitro propagation showed marked significance in almost all parameters under present investigation. Media without silver nitrate showed lesser response. This may be due to presence of silver nitrate, which might have suppressed the activity of excess of ethylene present in the in vitro culture tubes and further promoted for easy translocation and assimilation of these energy sources available in the media by the explants resulting in cell division and leading to vigorous growth. Similar type of results was documented in Decalepis hamiltonii [16], Vanilla planifolia [17].

Acclimatization and hardening

The well rooted shoots were removed from the culture tubes and washed thoroughly to remove the traces of agar. The plantlets of in vitro grown Solanum nigrum with well developed roots and shoots were transplanted to plastic cups containing autoclaved vermiculite and soil (1:1). About 90% of the transplanted plantlets survived after acclimatization and showed healthy growth without any morphological variations. Finally after one month the hardened plants were transferred to pots containing garden soil and sand (2:1) and were allowed to grow under nursery shade conditions. These plants were watered at 3 days intervals and were finally planted in field condition. All the plantlets were phenotypically indistinguishable from the parent plants.

CONCLUSION

Vegetative plant parts especially leaves are desirable explants for in vitro improvement because of regeneration from these explants would preserve the genetic homozygosity of the parent genotype. Callus culture system offer many advantages as a model system for several biological investigations. Hence in the present investigation a standardized protocol has been devised for in vitro callus induction and indirect shoot regeneration using ethylene inhibitor silver nitrate from young leaf explants of Solanum nigrum.

ABBREVIATIONS

2, 4-D	*2, 4-Dichloro phenoxy acetic acid*
$AgNO_3$	*Silver nitrate*
NAA	*α – naphthalene acetic acid*
IBA	*Indole – 3 – butyric acid*
IAA	*Indole – 3 – acetic acid*

Table-1: Effect of different concentrations of $AgNO_3$ and plant growth regulators on induction of callus from leaf explants of field grown Solanum nigrum plants.

Intensity of callus: C^{+} = very low; C^{++} = low; C^{+++} = high callus.

Plant growth regulators (mg/l)				Concentration of $AgNO_3$ (mg/l)	Intensity of callus formation	Nature of callus
NAA	2, 4-D	IAA	BAP			
1.0	-	-	0.5	-	+	Light brown, fragile
1.0	-	-	0.5	0.1	++	Light green, compact
1.0	-	-	0.5	0.2	++	Whitish green, hairy roots
1.0	-	-	0.5	0.4	+	Creamish yellow, compact
1.0	-	-	0.5	0.6	+	Yellowish green, fragile
2.0	-	-	0.5	-	+	Dark brown, nodular
2.0	-	-	0.5	0.1	++	Whitish yellow, nodular
2.0	-	-	0.5	0.2	+++	Creamish green, compact
2.0	-	-	0.5	0.4	++	Light brown, fragile
2.0	-	-	0.5	0.6	+	Dark green, compact
-	1.0	-	0.5	-	+	Light green, hairy roots
-	1.0	-	0.5	0.1	++	Yellowish green, nodular
-	1.0	-	0.5	0.2	++	Light brown, fragile
-	1.0	-	0.5	0.4	+	Light green, compact
-	1.0	-	0.5	0.6	+	Green, nodular
-	2.0	-	0.5	-	+	Brown, fragile
-	2.0	-	0.5	0.1	+	Light yellowish, nodular
-	2.0	-	0.5	0.2	++	Light green, compact
-	2.0	-	0.5	0.4	++	Dark brown, compact
-	2.0	-	0.5	0.6	+	Pale green, hairy roots
-	-	1.0	0.5	-	+	Dark green, compact
-	-	1.0	0.5	0.1	++	Brown, loose, fragile
-	-	1.0	0.5	0.2	++	Whitish green, nodular
-	-	1.0	0.5	0.4	+	Light yellowish green, nodular
-	-	1.0	0.5	0.6	+	Light green, fragile
-	-	2.0	0.5	-	+	Dark brown, fragile
-	-	2.0	0.5	0.1	+	Brown, loose, fragile
-	-	2.0	0.5	0.2	++	Light green, compact
-	-	2.0	0.5	0.4	++	Yellowish green, fragile
-	-	2.0	0.5	0.6	+	Light green, nodular

Table-2: Effect of different concentrations of $AgNO_3$ and plant growth regulators on induction of callus from nodal explants of field grown Solanum nigrum plants.

Intensity of callus: C^{+} = very low; C^{++} = low; C^{+++} = high callus.

Plant growth regulators (mg/l)				Concentration of $AgNO_3$ (mg/l)	Intensity of callus formation	Nature of callus
NAA	IAA	2, 4-D	BAP			
1.0	-	-	0.5	-	+	Yellowish green, fragile
1.0	-	-	0.5	0.1	+	Creamish, fragile
1.0	-	-	0.5	0.2	++	Light cream, compact
1.0	-	-	0.5	0.4	+	Brown, fragile
1.0	-	-	0.5	0.6	+	Whitish green, organogenic
2.0	-	-	0.5	-	+	Green, nodular
2.0	-	-	0.5	0.1	+	Light brown, profuse
2.0	-	-	0.5	0.2	++	Light green, organogenic
2.0	-	-	0.5	0.4	+	Creamish brown, fragile
2.0	-	-	0.5	0.6	+	Green, compact
-	1.0	-	0.5	-	+	Greenish white, fragile
-	1.0	-	0.5	0.1	++	Yellowish green, compact
-	1.0	-	0.5	0.2	++	Dark brown, fragile
-	1.0	-	0.5	0.4	+	Light cream, compact
-	1.0	-	0.5	0.6	+	Green, organogenic
-	2.0	-	0.5	-	+	Light yellowish, nodular
-	2.0	-	0.5	0.1	+	Light brown, compact
-	2.0	-	0.5	0.2	++	Green, organogenic
-	2.0	-	0.5	0.4	++	Whitish green, compact
-	2.0	-	0.5	0.6	+	Creamish brown, compact
-	-	1.0	0.5	-	+	Brown, fragile
-	-	1.0	0.5	0.1	++	Dark green, compact
-	-	1.0	0.5	0.2	++	Creamish green, nodular
-	-	1.0	0.5	0.4	+	yellowish green, fragile
-	-	1.0	0.5	0.6	+	Light yellow, fragile
-	-	2.0	0.5	-	+	Light brown, compact
-	-	2.0	0.5	0.1	+	Green, fragile
-	-	2.0	0.5	0.2	+++	Light greenish white, compact
-	-	2.0	0.5	0.4	++	Brown, nodular
-	-	2.0	0.5	0.6	+	Light green, fragile

Table-3: Effect of different concentrations of BAP, NAA and IAA in combination with $AgNO_3$ on indirect organogenesis from in vitro grown callus of Solanum nigrum. Observation: *After 8 weeks, values are mean ± S.E. of 10 independent determinants.*

Plant growth regulators (mg/l)			Concentration of $AgNO_3$ (mg/l)	Regeneration frequency (%)	Mean no. of shoots/callus	Mean shoot length (cm)
BAP	NAA	IAA				
1.0	0.1	-	-	55	2.8 ± 0.20	1.69 ± 0.08
1.0	0.1	-	0.4	70	4.4 ± 0.25	3.0 ± 0.17
1.0	0.5	-	-	65	3.3 ± 0.31	2.5 ± 0.28
1.0	0.5	-	0.4	75	7.0 ±0.36	3.7 ± 0.26
1.0	-	0.1	-	50	2.3 ± 0.13	3.1 ± 0.52
1.0	-	0.1	0.4	65	3.9 ± 0.36	4.2 ± 0.14
1.0	-	0.5	-	62	4.3 ± 0.15	3.5 ± 0.31
1.0	-	0.5	0.4	75	6.5 ± 0.32	4.9 ± 0.40
2.0	0.1	-	-	70	3.6 ± 0.29	3.5 ±0.19
2.0	0.1	-	0.4	80	8.8 ± 0.36	5.7 ± 0.33
2.0	0.5	-	-	78	5.5 ± 0.22	4.4 ± 0.36
2.0	0.5	-	0.4	90	15.6 ± 0.29	6.6 ± 0.25
2.0	-	0.1	-	65	2.5 ± 0.32	3.0 ± 0.56
2.0	-	0.1	0.4	70	7.4 ± 0.37	5.6 ± 0.87
2.0	-	0.5	-	75	4.9 ±0.25	4.4 ± 0.43
2.0	-	0.5	0.4	80	12.4 ± 0.30	6.13± 0.75
3.0	0.1	-	-	55	3.6 ± 0.28	2.44 ± 0.05
3.0	0.1	-	0.4	60	8.5 ± 0.1	3.61 ± 0.34
3.0	0.5	-	-	62	4.6± 0.43	3.0± 0.76
3.0	0.5	-	0.4	75	9.9 ± 0.26	4.9 ± 0.22
3.0	-	0.1	-	50	2.5 ± 0.14	1.55 ± 0.30
3.0	-	0.1	0.4	55	6.5 ± 0.32	3.2 ± 0.31
3.0	-	0.5	-	60	3.3 ± 0.1	2.8 ± 0.24
3.0	-	0.5	0.4	65	7.6 ± 0.30	4.9 ± 0.25

Table-4: Effect of $AgNO_3$ on different concentrations of IBA, NAA and IAA on in vitro rooting using half strength MS medium. Observation: *After 8 weeks, values are mean ± S.E. of 20 independent determinants.*

Plant growth regulators (mg/l)			Concentration of $AgNO_3$ (mg/l)	Regeneration frequency (%)	Mean no. of roots/shoot	Mean root length (cm)
IBA	NAA	IAA				
1.0	-	-	-	80	15.4 ± 0.39	3.69 ± 0.26
1.0	-	-	0.4	90	28.6 ± 0.25	4.85 ± 0.10
2.0	-	-	-	85	17.7 ± 0.32	4.26 ± 0.17
2.0	-	-	0.4	98	39.4 ± 0.33	5.4 ± 0.39
3.0	-	-	-	75	13.9 ± 0.26	2.73 ± 0.15
3.0	-	-	0.4	85	25.7 ± 0.89	3.4 ± 0.29
-	1.0	-	-	75	11.6 ± 0.3	2.2 ± 0.51
-	1.0	-	0.4	85	22.4 ± 0.39	4.61 ± 0.33
-	2.0	-	-	82	13.8 ± 0.47	3.1 ± 0.66
-	2.0	-	0.4	95	27.5 ± 0.2	3.65 ± 0.28
-	3.0	-	-	72	10.7 ± 0.26	1.4 ± 0.15
-	3.0	-	0.4	80	20.3 ± 1.30	2.9 ± 0.65
-	-	1.0	-	65	9.5 ± 0.37	1.70 ± 0.27
-	-	1.0	0.4	75	16.7 ± 0.16	3.6 ± 0.22
-	-	2.0	-	70	12.3 ± 0.30	2.79 ± 0.36
-	-	2.0	0.4	80	20.1 ± 0.52	4.26 ± 0.81
-	-	3.0	-	62	9.4 ± 1.04	2.3 ± 0.78
-	-	3.0	0.4	78	14.4 ± 0.14	2.39 ± 0.44

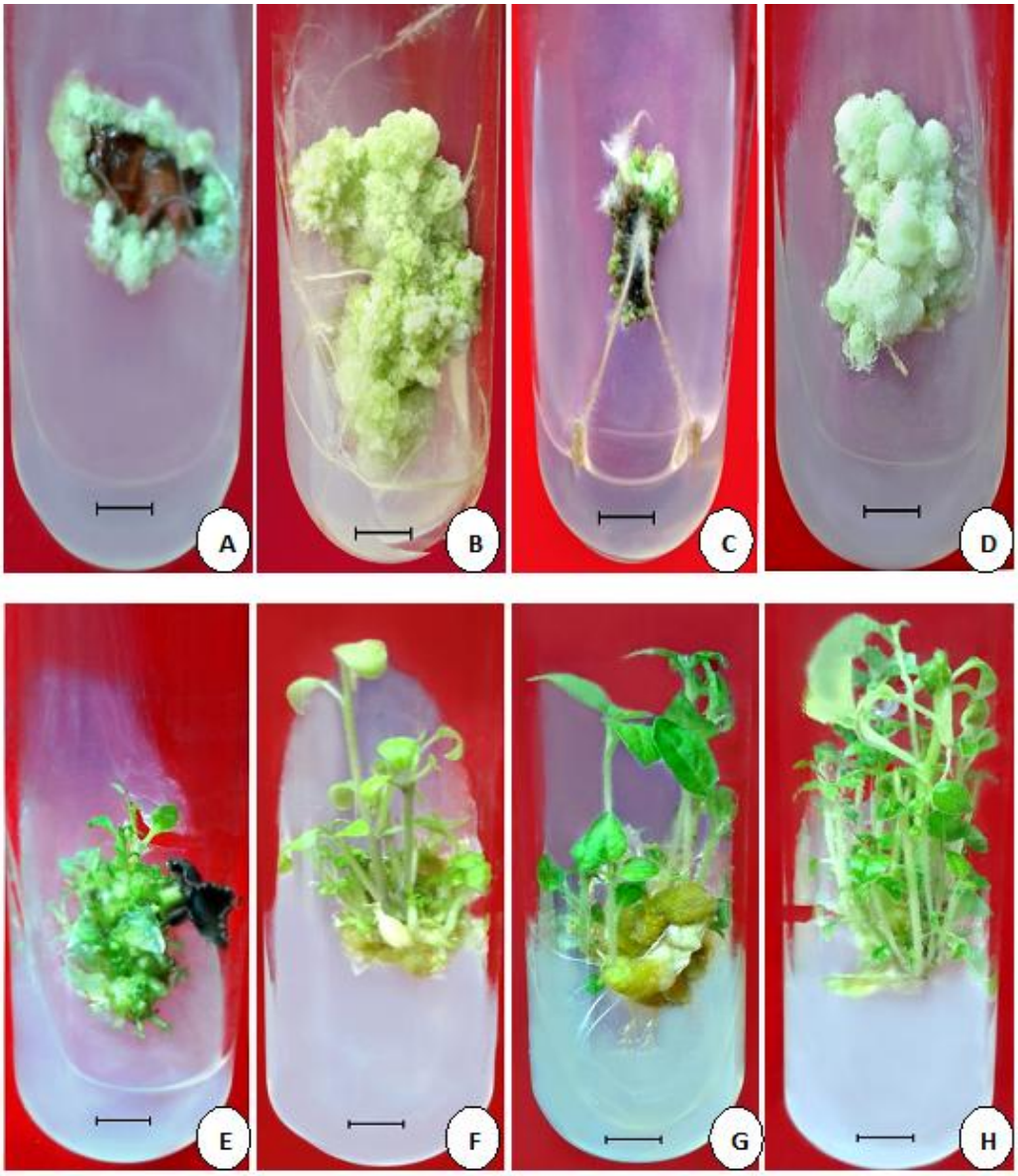

Fig 1: Callus formation from young leaf and nodal explants of field grown Solanum nigrum Callus formation from leaf explants of field grown plants

A) NAA (1.0 mg/l) + BAP (0.5 mg/l) and $AgNO_3$ (0.1 mg/l), B) NAA (2.0 mg/l) + BAP (1.0 mg/l) and $AgNO_3$ (0.2 mg/l)

Callus formation from nodal explants of field grown plants

C) 2, 4- D (1.0 mg/l) + BAP (0.5 mg/l) and $AgNO_3$ (0.1 mg/l) D) 2, 4- D (2.0 mg/l) + BAP (0.5 mg/l) and $AgNO_3$ (0.2 mg/l)

Indirect adventitious shoot regeneration from in vitro derived calli

E) MS + BAP (1.0 mg/l) + NAA (0.1 mg/l) and $AgNO_3$ (0.4 mg/l), F) MS + BAP (2.0 mg/l) + NAA (0.1 mg/l) and $AgNO_3$ (0.4 mg/l), G-H) MS + BAP (2.0 mg/l) + NAA (0.5 mg/l) and $AgNO_3$ (0.4 mg/l)

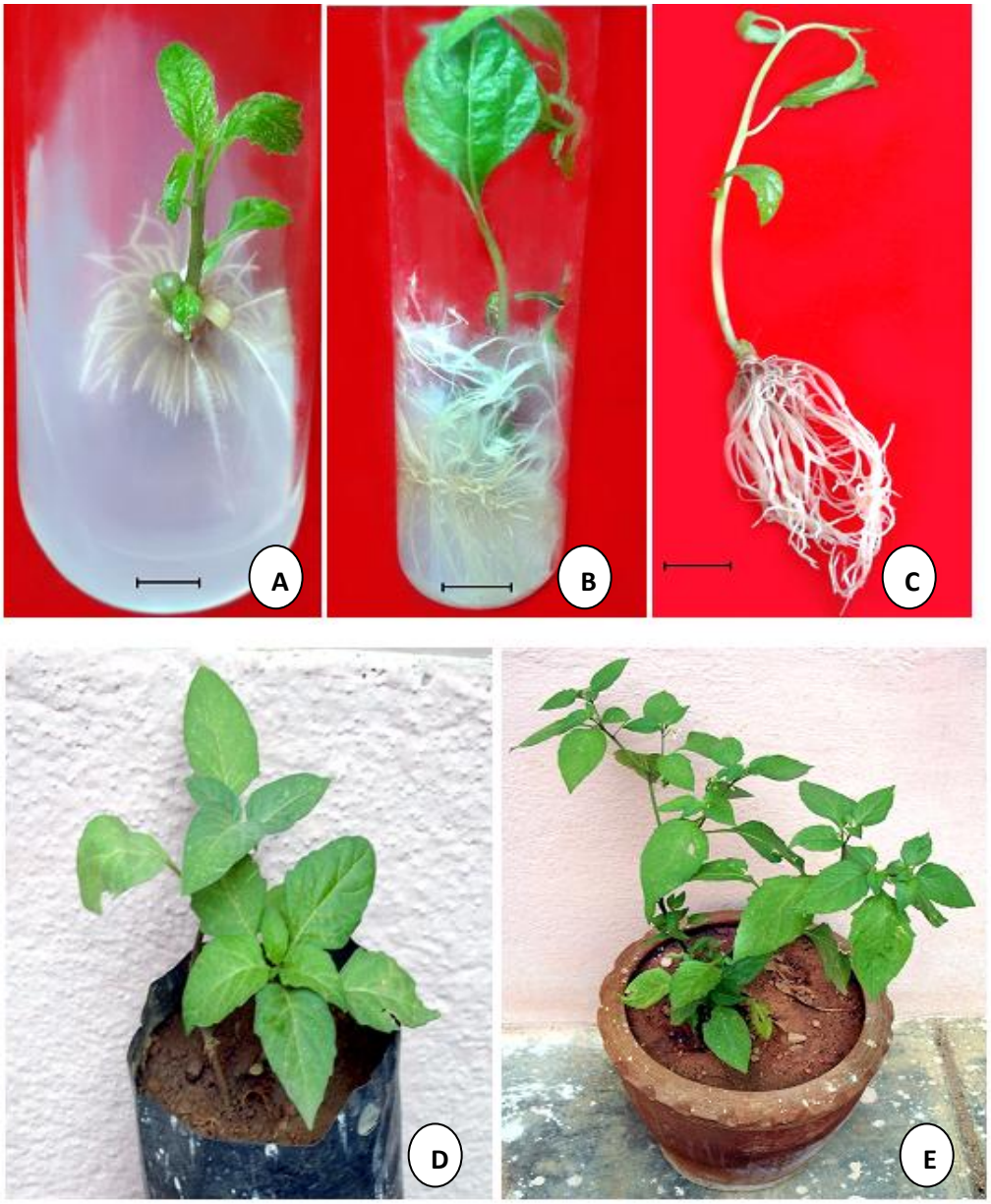

Fig-2: Rooting and acclimatization of Solanum nigrum: *A, B) Root formation from shootlets inoculated on MS media with IBA (1.0 mg/l) and AgNO3 (0.4 mg/l) C) Plantlet showing elongated root system D) Hardened plantlet in polybags containing soil and vermiculate in 1:1 ratio E) Plantlet in field condition*

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