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Influence of Plant Growth Regulators (PGRs), Antioxidants, $HgCl_2$ and photoperiod on *in vitro* shoot proliferation of Ashwagandha (*Withania somnifera*)– a medicinal crop

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Abstract

The present investigation was carried out to see effects of Plant Growth Regulators, antioxidants, $HgCl_2$ and photo period on various characters of shoot proliferation *viz*., period of initiation of shoots, response (%), number of shoots explants^{-1}, number of nodes explant^{-1} and shoot length (cm) with objectives to optimize conditions of proliferation from nodal explants. Callus induction and indirect regeneration was also studied. MS basal media supplemented with combination of cytokinin and auxin *viz*., (1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} BAP + 2.0 mg l^{-1} NAA, 2 .0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA and 1 .0 mg l^{-1} Kinetin + 1.0 mg l^{-1} IAA, 1.0 mg l^{-1} Kinetin + 2.0 mg l^{-1} IAA, 2 .0 mg l^{-1} Kinetin + 1.0 mg l^{-1} IAA). Maximum shoots were induced with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, showed highest (85.33%) response of shooting. All the treatment combinations of BAP and NAA were found most responsive as compared to Kinetin and IAA. When activated charcoal (100-300 mg l^{-1}) and ascorbic acid (50-150 mg l^{-1}) were added in medium supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA. 200 mg l^{-1} of activated charcoal and 100 mg l^{-1} of ascorbic acid showed highest (80.00% and 80.40%) response of shoot induction. In photo period treatment, maximum shoot induction (2.57) was observed in 16:8 hours with (100%) response with minimum days shooting and maximum number of shoot regeneration (3.71) from callus with highest morpho genesis response. Maximum contamination control (89.14%) along with maximum shoot induction (3.86) was observed when explant was sterilized with 0.1% $HgCl_2$ for 3 minutes.

Keywords: Ashwagandha, Plant Growth Regulators (PGRs), $HgCl_2$, photo period, shoot proliferation, NAA: (Naphthalene acetic

acid), BAP (6-Benzylaminopurine)
IAA (Indole-3-acetic acid)

Introduction

Ashwagandha (*Withania somnifera* L.) is a potential medicinal plant belonging to Solanaceae family. It has gained a lot of attention since ancient times due to its immense pharmaceutical potential (Singh *et al*., 2016). It has a high reputation among different phytotherapeutics due to its multiple pharmacological properties such as immunomodulatory, neuroprotective, cardioprotective, bone healing, anti-cancer, anti-ageing, anti-inflammatory, anti-oxidant, and anti-apoptotic (Dar *et al*., 2015; Kaur *et al*., 2017). More than 12 types of alkaloids and 35 different with anolides have been recorded from this plant (Verma and Kumar, 2011). The production of dried plant material of *W. somnifera* for with anolides is estimated to be about 5905 tonnes per year against the annual requirement of 9127 tonnes (Rangaraju *et al*., 2018).

With the increasing demand for *W. somnifera* in larger quantities, it is cultivated almost as a commercial crop in India which is propagated from seeds, but the mature and healthy seeds are not always available for germination. The production of *W. somnifera* through conventional methods of cultivation (seed) is less than the requirement due to numerous reasons *viz*. poor yield, takes long time, low viability of seeds (limited to one year), percentage of seed germination due to the presence of certain inhibitory substances, low seedling survival rate, susceptibility of the seeds and seedlings to fungal infections like seedling mortality and blight, leaf blight, seed rotting etc (Kaur *et al*., 2017; Singh *et al*., 2017). This medicinally significant plant species has been depleted from its natural habitat and is now included in the list of endangered species (Patel and Krishna murthy 2013) by the International Union for Conservation of Nature and Natural Resources (Supe *et al.,* 2006). The rapid multiplication of *W. somnifera* by tissue culture techniques can help to solve these problems.

Large scale production through plant *in vitro* regeneration will provide a means of putting the plant onto the market at lower prices. Studies have reported *in vitro* propagation of Ashwagandha by using different explants, such as shoot tips (Rani *et al*., 2014), axillary bud, hypocotyl, cotyledon, leaf, leaf segments, callus of leaf, shoot tip and root and the nodal areas. The available literature on *W. somnifera* suggests that the plant responds differently to different culture conditions and plant growth regulators (PGRs). In most reports, 6-benzylaminopurine (BAP) is favourably used for optimum shoot proliferation and indole-3-butyric acid (IBA) for in vitro rooting (Saema *et al*., 2015; Singh *et al*., 2016).

In this background, the establishment of an effective *in vitro* propagation system for *W. somnifera* offers an attractive alternative that would facilitate rapid multiplication and production of genetically as well as phytochemically uniform plant material for pharmaceutical industries. Considering these problems and limitations, present study has been undertaken to explore potential of Plant Growth Regulators, $HgCl_2$ and photo period on *in vitro* shoot proliferation of Ashwagandha (*Withania somnifera*).

Material and Methods

***Media preparation and culture conditions*:** The present research was carried out at the Plant Tissue Culture Laboratory situated at Department of Genetics and Plant Breeding, S.K.N. College of Agriculture, Jobner, Rajasthan during year 2023. All chemicals used in the present study were of analytical grade. Murashige and Skoog (1962) medium was used throughout the course of investigation. A beaker was filled with 800 ml of double distilled water. 4.4 g of MS medium (powdered), 30 g of sucrose, and 8 g of agar were added to the beaker and heated to mix well. Plant growth regulators were added to the basal medium at the end before autoclaving. The pH of the medium was adjusted at 5.84 using 1N NaOH or 1N HCl solutions. Volume was made up to 1 litre. Culturing flask and test tubes were filled with media. The culture media contained in a conical flask and test tubes sealed with cotton plugs and covered with aluminum foils were autoclaved at 15 psi and 121°C for 30 minutes. Exposure time depends on the volume of the liquid to be sterilized. After autoclaving, media were stored in the dark for 48 hours at 25±2°C. Different concentrations of treatments were incorporated in combinations in the MS medium for shoot proliferations

are as follows: Plant growth regulators incorporated in combinations. (a) BAP (1.0 and 2.0 mg l^{-1}) + NAA (1 and 2.0 mg l^{-1}). (b) Kinetin (1.0 and 2.0 mg l^{-1}) + IAA (1.0 and 2.0 mg l^{-1}), activated charcoal (100-300 mg l^{-1}) and ascorbic acid (50-150 mg l^{-1}), photo period regimes (16:8; 14:10; 12:12; 8:16 hours) and time of sterilization with 0.1% $HgCl_2$.

Inoculation of explants: After sterilization, the explants were inoculated on culture media aseptically. For inoculation, explants were transferred to a large sterile glass petriplates with the help of sterile forceps under strict aseptic conditions. Here, the explants were further trimmed to desired size upto 2-3 cm with sterile scalpel blade. After cutting explants of suitable size, these were transferred to culture test tubes and borosil flasks containing MS medium supplemented with different plant growth regulators. After vertically inoculating the explants in the culture test tubes and borosil flasks, mouth of the test tubes and borosil flasks were quickly flamed and were closed with non-adsorbent cotton plug. All cultures were incubated at 25±2°C under fluorescent light for 16: 8 hour's photo period.

Data recording and analysis

The experiment was conducted in completely randomized design with each treatment combination was replicated 10 times. Cultures were observed periodically and following observations were recorded: period of initiation of shoots response (%), number of shoots explants^{-1}, number of nodes explant^{-1} and shoot length (cm), callus weight, days taken in regeneration, days taken in callus induction, browning Intensity. Data were analyzed for mean and standard error.

Results

Effect of different concentration of Plant Growth Regulators (PGRs)

Nodal segments explants were inoculated on MS basal media supplemented with combination of cytokinin and auxin at different level of concentration (1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, 1.0 mg l^{-1} BAP + 2.0 mg l^{-1} NAA, 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kinetin + 1.0 mg l^{-1} IAA, 1.0 mg l^{-1} Kinetin + 2.0 mg l^{-1} IAA, 2.0 mg l^{-1} Kinetin + 1.0 mg l^{-1} IAA) to see their effects on various the characters *viz*., period of initiation of shoots, response (%), number of shoots/explant, number of nodes/ explant and shoot length (cm) (Plate 1).

Out of three combination treatment of BAP and NAA, 2 .0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA showed highest (85.33%) response of shoot induction within minimum (15.33) days of inoculation with maximum number of shoot per explant (4.00) with highest nodes per explants (4.67) and shoot length (7.83 cm) (Table 1). Lowest response (61.67%) of shoot induction with minimum number of shoot per explant (2.33) with lowest nodes per explants (2.67) and shoot length (6.35) was observed with 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA concentration, whereas maximum days for initiation of shoot (18.00) was observed with 1 .0 mg l^{-1} BAP + 2.0 mg l^{-1} NAA combination treatment.

In combination treatment of Kinetin and IAA, 2.0 mg l^{-1} Kinetin + 1.0 mg l^{-1} IAA showed highest (80.33 %) response of shoot induction within minimum (16.00) days of inoculation with maximum number of shoot per explant (2.67) with highest nodes per explants (3.67) and shoot length (7.33) (Table 1). Lowest response (55.00%) of shoot induction with minimum number of shoot per explant (1.33) with lowest nodes per explants (2.00) and shoot length (6.10 cm) was observed with 1.0 mg l^{-1} Kinetin + 1.0 mg l^{-1} IAA concentration, whereas maximum days for initiation of shoot (20.67) was observed with 1.0 mg l^{-1} Kinetin + 2.0 mg l^{-1} IAA combination treatment.

Plate 1: *In vitro* propagation of *Withania somnifera* (a) Explant sterilization (b) Shoot induction (c) Indirect regeneration (d) Callus growth

Table 1. Effect of different concentration of Plant Growth Regulators (PGRs) incorporated in combinations (cytokinin + auxin) in MS media on shoot proliferation in Ashwagandha

Plant Growth Regulators (PGRs)	Period of initiation of shoots	Response (%)	Number of shoots explants $^{-1}$	Number of nodes explant $^{-1}$	Shoot length (cm)
BAP + NAA (mg/l)					
1 .0 + 1.0	17.67	61.67 (51.75)	2.33 (1.68)	2.67 (1.77)	6.35
1.0 +2.0	18.00	79.00 (62.73)	2.67 (1.77)	3.33 (1.95)	6.50
2.0 +1.0	15.33	85.33 (67.5)	4.00 (2.12)	4.67 (2.27)	7.83
Kinetin + IAA (mg/l)					
1 .0 + 1.0	19.00	55.00 (47.87)	1.33 (1.34)	2.00 (1.58)	6.10
1.0 +2.0	20.67	71.67 (57.85)	1.33 (1.34)	2.33 (1.68)	6.13
2.0 +1.0	16.00	80.33 (63.67)	2.67 (1.77)	3.67 (2.04)	7.33
S.Em.	0.47	0.58	0.10	0.08	0.18
C.D. (5%)	1.46	1.80	0.31	0.25	0.56
CV (%)	4.60	1.72	10.36	7.51	4.71

In comparison of treatments, all the treatment combinations of BAP and NAA combination was found most responsive as compared to Kinetin and IAA for parameters of shoot proliferations.

Effect of Antioxidants (activated charcoal)

When activated charcoal (100-300 mg l^{-1}) was added in basal medium with micro propagation protocol from nodal segment supplemented with 2.0 mg l^{-1} BAP + 1.0 mg/l NAA, it induces shoot and callus at all levels. 200 mg l^{-1} of activated charcoal showed highest (80.00%) response of shoot induction within minimum (16.00) days of induction of shoots with maximum number of shoot per explant (2.90). Whereas, 300 mg l^{-1} of activated charcoal showed lowest (60.00%) response of shoot induction within maximum (18.00) days of inoculation with minimum number of shoot per explant (2.38). Days taken in callus induction were significantly lower (23.18) and maximum callus proliferation efficacy (1.06 g) was significantly higher when medium supplemented with 200 mg l^{-1} of activated charcoal than all the treatment. Treatments above and below 200 mg l^{-1} revealed gradual declination in callus weight and increased days to callus induction. 200 mg l^{-1} of activated charcoal was able to control accumulation of phenolic compound as medium showed low browning intensity. All other level showed medium browning intensity (Table 2).

Table 2. Effect of different concentration of antioxidants (activated charcoal) incorporated in in MS media supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA on shoot proliferation and callus induction in Ashwagandha

Activated charcoal (mg l^{-1})	Period of initiation of shoot	Response (%)	Number of shoots explants $^{-1}$	Days taken in callus induction	Callus weight (gm)	Browning Intensity
100	17.80	65.40 (54.012)	2.60 (1.76)	27.58	0.94	++
200	16.00	80.00 (63.43)	2.90 (1.84)	23.18	1.06	+
300	18.00	60.00 (50.77)	2.38 (1.7)	28.68	0.95	++
S.Em.	0.49	0.79	0.03	0.46	0.01	
C.D. (5%)	1.52	2.43	0.09	1.42	0.02	
CV (%)	4.95	3.15	3.58	4.09	1.76	

(++) Medium browning, (+) low browning

Effect of Antioxidants (ascorbic acid)

Incorporation of ascorbic acid (50-150 mg l^{-1}) in MS medium supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA induces shoot at all levels and callus at all levels. Maximum response of shoot induction (80.40%) was observed with 100 mg l^{-1} of ascorbic acid with minimum (17.76) days of induction of shoots with maximum number of shoot per explant (2.76). Whereas, 50 mg l^{-1} of ascorbic acid showed lowest (54.20%) response of shoot induction within maximum (18.84) days of inoculation with minimum number of shoot per explant (2.32). Days taken in callus induction were significantly lower (25.12) with maximum callus proliferation efficacy (1.03 g) was significantly higher when medium supplemented with 100 mg l^{-1} of ascorbic acid than all the treatment. Treatments above and below 100 mg l^{-1} revealed gradual declination in callus weight and increased days to callus induction. 100 mg l^{-1} of ascorbic acid was able to control accumulation of phenolic compound as medium showed low browning intensity. All other level showed medium browning intensity. 50 mg l^{-1} of ascorbic acid was insufficient to control the accumulation of phenolic compound as medium showed intense browning. Activated charcoal efficiently raise the parameters of shoot proliferation and callus induction while ascorbic acid produces less number of shoots, more days for shoot and callus induction and less control over browning of medium under similar culture conditions (Table 3; Plate 1).

Effect of photo period regimes on shoot proliferation

In the present investigation different photo period regimes (16:8; 14:10; 12:12; 8:16 hours) were assessed on shoot proliferation in MS media supplemented with 2.0 mg l^{-1} BAP. Maximum shoot induction (2.57) was observed in 16:8 hours photo period with (100%) responsive with minimum days of shoot induction (18.57) and highest shoot length (6.69 cm) followed by 14:10 hours with minimum shoot induction (1.00) with (97.14%) responsive with maximum days of shoot induction (21.71) and lowest shoot length (5.57 cm). 12:12; 8:16 hours photo period was insufficient to induce shoot bud even on responsive level of plant growth regulators. Significant difference was observed in shoot bud induction at different photo period regimes (Table 4a).

Effect of photo period regimes on indirect regeneration

Indirect shoot regeneration from callus culture exhibited significant difference at different photo period regimes in MS media supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA. Maximum number of shoot regenerated (3.71) was observed with highest morphogenesis response (81%) and less days taken for regeneration (32.14) in 16:8 hours photo period followed by 14:10 hours where minimum number of shoot regenerated (2.43) with low morphogenesis response (51.71%) and more days taken for regeneration (38.14). 12:12; 8:16 hours photo period was insufficient to induce regeneration on responsive level of plant growth regulators. Differences in regeneration were significant at different photoperiod regimes (Table 4b).

Effects of time of sterilization with 0.1% $HgCl_2$

In the present investigation, effects of time period of sterilization with 0.1% $HgCl_2$ (1, 3, 5 minutes) were assessed on reduction in contamination and shoot proliferation in MS media supplemented with 2.0 mg l^{-1} BAP. Maximum contamination control (89.14%) was observed when explant was sterilized with 0.1% $HgCl_2$ for 3 minutes and lowest (53.57%) in 1 minute treatment.

Maximum shoot induction (3.86) was observed when explants were sterilized in 0.1% mercuric chloride for 3 min with minimum days of shoot induction (17.57) and highest shoot length (7.79 cm) followed by explants sterilized with 0.1% mercuric chloride for 1 min where less number of shoot induction (2.29), more days of shoot induction (18.00) and less shoot length (6.39 cm) was observed. Significant difference was observed in shoot bud induction at different treatments. 0.1% $HgCl_2$ for 5 minutes was insufficient to contamination control and induces shoot bud even on responsive level of plant growth regulators where al plants were burned. The contamination rate was still higher when sterilized in 0.1% mercuric chloride for 1 min than 3 min (Table 5).

Table 3. Effect of different concentration of antioxidants (ascorbic acid) incorporated in MS media supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA on shoot proliferation and callus induction in Ashwagandha

Ascorbic acid (mg l^{-1})	Period of initiation of shoot	Response (%)	Number of shoots explant^{-1}	Days taken in callus induction	Callus weight (gm)	Browning Intensity
50	18.84	54.20 (47.41)	2.32 (1.68)	28.60	0.83	+++
100	17.76	80.40 (63.72)	2.76 (1.81)	25.12	1.03	+
150	19.40	71.20 (57.55)	2.38 (1.7)	27.36	0.94	++
S.Em.	0.20	0.33	0.18	0.01	0.01	
C.D. (5%)	0.62	1.02	0.55	0.03	0.02	
CV (%)	1.86	1.32	1.48	1.29	1.86	

(+++) Intense browning, (++) medium browning, (+) low browning

Table 4a. Effects of photoperiod regimes on shoot proliferation in MS media supplemented with 2.0 mg l^{-1} BAP

Photoperiod regimes (hours)	Period of initiation of shoot	Response (%)	Number of shoots explants $^{-1}$	Shoot length (cm)
16:8	18.57	100.00 (90)	2.57 (1.75)	6.69
14:10	21.71	97.14(84.73)	1.00 (1.22)	5.57
12:12	-	-	-	-
8:16	-	-	-	-
S.Em.	0.40	2.40	0.04	0.15
C.D. (5%)	1.22	7.41	0.12	0.45
CV (%)	5.21	7.28	6.73	6.32

(-) No response

Table 4b. Effects of photoperiod regimes on indirect regeneration from callus in MS media supplemented with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA

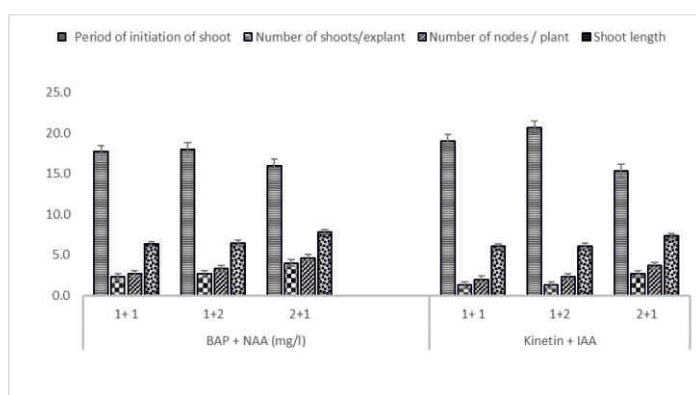
Photoperiod regimes (hours)	Days taken in regeneration	Morphogenesis response (%)	Number of shoot regenerated
16:8	32.14	81.00	3.71(2.05)
14:10	38.14	51.71	2.43(1.71)
12:12	-	-	-
8:16	-	-	-
S.Em.	0.34	0.61	0.05
C.D. (5%)	1.05	1.89	0.16
CV (%)	2.56	2.44	7.53

(-) No response

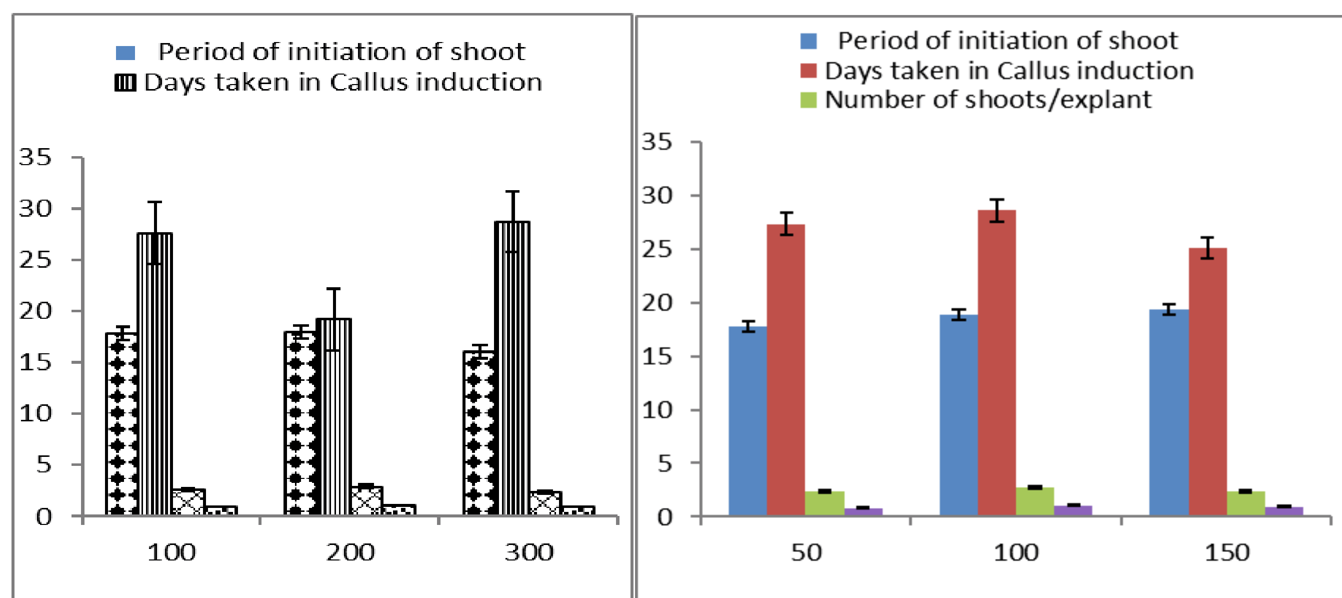
Table 5. Effects of time of sterilization with 0.1% $HgCl_2$ on shoot proliferation in MS media supplemented with 2.0 mg l^{-1} BAP

Effects of time of sterilization with 0.1% $HgCl_2$	Period of initiation of shoot	Per cent of reduction in contamination	Number of shoots explants $^{-1}$	Shoot length (cm)
1 minute	18.00	53.57 (47.05)	2.29 (1.66)	6.39
3 minutes	17.57	89.14 (70.8)	3.86 (2.08)	7.79
5 minutes	-	-	-	-
S.Em.	0.26	0.51	0.07	0.10
C.D. (5%)	0.81	1.58	0.20	0.31
CV (%)	3.90	2.31	9.26	3.73

(-) No response



Graph 1: Effect of different concentration of Plant Growth Regulators on shoot proliferation in Ashwagandha



Graph 2: Effect of different concentration of antioxidants (activated charcoal) (ascorbic acid) incorporated in MS media on shoot proliferation and callus induction in Ashwagandha

Discussion

In the current investigation high cytokinin to auxin ratio enhanced shoot multiplication considerably. The adding of BAP was more effective to increasing the number of shoots than Kinetin. Multiplication of shoots on MS media added by 2 .0 mg l^{-1} BAP gave the best results with highest shoot per explant (4.00). The effectiveness of BAP compared to Kinetin has also been reported by Royani *et al*., 2021. Similar results were obtained by Baksha *et al*., 2005, Kian *et al*., 2017, Dixit *et al*., 2020 and Ahmad *et al*., 2023 who reported synergistic effects of BAP and NAA on shoot multiplication (Table 1).

One of the major problems associated with plant tissue culture is browning of the culture medium and the explants, which invariably leads to death of plants. The browning of the medium is due to releasing phenol by the explants which get oxidized, and this oxidation product could be phytotoxic. Thus, it needs a scrutinized investigation before incubating explants in the culture medium In order to control browning of medium and explants in vitro, many workers have tried to incorporating nonspecific absorbents like activated charcoal, ascorbic acid and polyvinylpyrrolidone in to the culture medium (Weatherhead *et al*., 1979 and Bharadwaj and Ramawat, 1992).

In the present investigation two different antioxidants (activated charcoal and ascorbic acid) were incorporated singly in MS medium supplemented with responsive level of plant growth regulators, elicited different response for shoot bud induction and callus differentiation because it controls the accumulation of inhibitory substances (phenolic compounds) in the growth medium. Among the two antioxidants, activated charcoal was found better in reducing browning of medium and explant. The results of present investigation for use of activated charcoal were in close agreement with observation of antioxidant by Aparna *et al*., 2017a, Kumar *et al*., 2018, Verma, 2019 and Ahmad *et al*., 2023.

Photo periodism can be defined as the developmental response of plants to the relative length of light and dark periods. Hence it should be emphasized that photo periodic effect relates directly to both light and dark periods. Photo periodism is one of the most significant and complex aspects of the interaction between plants and their environment. In the present investigation, different photo period regimes (16:8; 14:10; 12:12; 8:16 hours) were assessed on shoot proliferation in MS media supplemented with 2.0 mg l^{-1} BAP. Maximum shoot induction parameters and regeneration from callus was observed at in 16:8 hours photo period followed by 14:10 hours. 12:12; 8:16 hours photo period was insufficient to induce shoot bud even on responsive level of plant growth regulators. The response was best when dark period was shorted and reverse when dark period was longer. Similar results were observed by Jakhar *et al*., 2020, Aparna *et al*., 2017b, Burdak *et al*., 2017, Kumar, 2018, Ahmad *et al*., 2023, Aparna *et al*., 2023.

Mercury chloride is a commonly used effective surface fungicide for explant sterilization. Sterilization with $HgCl_2$ affected not only the contamination but culture establishment etc. The moderate time period of mercuric chloride while decreasing the rate of contamination, reduced browning and death of the explants. Mercury chloride has a certain toxic effect on plants, and this effect tends to be more pronounced with increasing treatment time. In the present investigation, effects of time period of sterilization with 0.1% $HgCl_2$ (1, 3, 5 minutes) were assessed on contamination and shoot proliferation reduction in MS media supplemented with 2.0 mg l^{-1} BAP. Maximum contamination control and shoot proliferation was observed when explant was sterilized with 0.1% $HgCl_2$ for 3 minutes. This result was in agreement with Verma *et al*., 2019 and Gu *et al*., 2022.

Conclusion

From the above-mentioned experiment, it may be concluded that optimum concentration of auxin and cytokinin for shoot proliferation of *Withania somnifera* was 2 .0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA and 2.0 mg l^{-1} Kinetin + 1.0 mg l^{-1} IAA. In the medium supplemented with various concentrations of antioxidants, 200 mg l^{-1} of activated charcoal and 100 mg l^{-1} of ascorbic acid showed highest shoot induction. 16:8 hours of photo period regimes induces maximum shoot highest morphogenesis from callus. Sterilization of explants with 0.1% $HgCl_2$ for 3 minutes was best in contamination control and shoot induction.

Conflict of interest: The authors declare no conflict of interest.

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