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Dedifferentiation And Redifferentiation Attempts In *Withania Somnifera*

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Abstract
The *Withania somnifera* is a medicinal herbaceous plant and has been explored extensively for the extraction of alkaloids having medicinal properties. Various growth regulating conditions were worked out in *Withania somnifera*. In the present work for dedifferentiation and redifferentiation an array of growth hormones were used. For callus formation NAA and KN combination was found to be most suitable in 10:1. For redifferentiation leaf primordial formation was observed maximum in 0.6 ml/L BAP. The shoot formation was observed in 0.5 ml/L Kinetin and in 4:1 ratio of NAA + BAP. The root formation was observed to be maximum in 20:1 NAA + K.
Key Words: *Withania somnifera*, dedifferentiation, callus formation, redifferentiation, leaf primordial formation, shoot formation, root formation.

INTRODUCTION

The vernacular name of *Withania* is ashwagandha also known as Indian Ginseng. **Ashwa** means horse and gandha means fragrance. The biological name of the plant is *Physalis flexuosa* and its family is solanaceae. Two species have been reported *W. coagulans* and *W. somnifera*. The chromosome number in *Withania somnifera* has been found to be 24 and 48. It is one of the 36 important cultivated medicinal plants. The plant has a large number of steroidal lactones which gives it importance (Palliyaguru *et al*., 2016). The problem with it is poor seed germination and viability.

The objective of the present project was to explore various *in vitro* methods in the genus *Withania* which will be helpful in large scale production of plants, though a number of attempts have been during past years on *in vitro* plant regeneration protocols (Siddhique et al., 2004; Supe *et al*. 2006; Wadegaonkar *et al*. 2006; Joshi and Padhya, 2010; Kumar *et al*. 2011; Rout *et al*. 2011; Saema *et al*. 2015; Kannan and Anbazhakan. 2016). Rani *et al*. (2003) reported callus induction and plantlet regeneration in *Withania somnifera.* Antonisamy and Manicham (1999) made attempts to conserve the withania species through *in vitro* propagation and restore them in the natural habitats. Manicham *et al.* (2000) regenerated Indian ginseng plantlets from stem callus. Furmanowa *et al*. (2001) produced *in vitro* plantlets from the shoottip of aseptically germinated seedlings. They also worked out the influence of elicitors on withaferin A production in tissue culture of *Withania*. Kulkani *et al*. (2000) studied rapid micropropagation of selected chemotypes using nodes, internodes, hypocotyls and embryo explants.

MATERIALS AND METHODS

In the present investigation potted plants were used. These plants were procured from forest research center Haldwani. The plant parts like leaf and stem, root, fruits and meristems were used as explants. The pots were placed in open environment. The objective of the present research was to work out different dedifferentiation and redifferentiation processes in Withania. For this different explants were taken as well as different growth hormones were used in various combinations. Standard tissue culture techniques were used as given below.

1. Explant preparation.

2. Tissue culture media preparation.
3. Methodology.
4. Sterilization of Medium.
5. Aseptic conditions.
6. Surface sterilization of plant material.
7. Inoculation.
8. Incubation and 9. Observations.

The *Withania* belongs to family Solanaceae. The plant body consists of well-developed roots, stem and leaves. The leaves were used as explants. In present work MS medium was used. The pH of the medium was adjusted to about 5.8.

Seven stock solutions (A, B, C, D, E, F, G) of 200 ml containing minerals and organic adjuvant were first prepared (plate 2 b). Each and every time 20ml of these stock solutions were taken for preparing a liter of MS medium. Sucrose and agar were dissolved in DDW separately and then the final volume was raised to 1000ml. The composition of stock solutions is given in table 1.

The medium after adding agar was poured in test tubes and flasks and autoclaved (15 min at 15 psi, 121^{o}C). Around 1 cm^2 of plant tissue was cut with a sharp blade. These explants were first washed with soapy water and running water for 15-20 minutes, and then transferred to a mercuric chloride solution (0.1%) for 5-6 minutes and then washed with DDW to remove any trace of $HgCl_2$. When the explant was surface sterilized, it was rinsed several times in sterile, distilled water. This last step was performed inside the laminar flow hood to maintain the aseptic condition of the explant and to prevent the reintroduction of contaminating microbes.

DDW and the implements such as scalpel, forceps, needles etc. were irradiated by ultraviolet germicidal light for 30 min before starting the inoculation and sub culturing procedures. All the implements were also sterilized by keeping them immersed in 90% alcohol and flamed on the spirit lamp before use. Inoculation was carried out in laminar flow and all precautions were taken while doing this experiment. The vessels were properly labeled.

For dedifferentiation two auxins 2, 4-D and NAA were used with cytokinins BAP and Kinetin (KN). These hormones were used in different proportions of 1:1, 4:1, 10:1 and 20:1 proportion.

For leaf development MS was used with BAP and KN in different concentrations 0.2, 0.4, 0.5, 0.6, 0.8 0.9 and 1.0 ml /L. For shoot development study MS was added with KN in 0.2, 0.3, 0.4 and 0.5 ml/L, both NAA and BAP were also used in combinations 1:1, 2:1, 3:1 and 4:1. For root development MS was added with BAP in concentration of 0.2 and 0.4 ml/L.

Now the culture vessel containing the explant was placed in controlled environment in incubator at 24±1^{o}C. The amount (quantity) of light, the spectral quality, the periodicity and the temperature (range and periodicity) all was optimum to the explants.

Table 1. The composition of seven stock solutions MS (Murashige and Skoog, 1962) medium containing nutrients.

Stock solution	Constituents	Concentration (g/200 ml)	Volume of stock solutions (ml)
A	NH_4NO_3	16.5	20
B	KNO_3	19.0	20
C	H_3BO_3	0.062	20
	KH_2PO_4	1.70	
	$NaMo.O_4$	0.0025	
	KI	0.0083	

Stock solution	Constituents	Concentration (g/200 ml)	Volume of stock solutions (ml)
	$CoCl_2.6H_20$	0.00025	
D	$CaCl_2.6H_2O$	4.40	20
E	$MgSO_4.7H_2O$	3.70	20
	$MnSO_4.4H_2O$	0.223	
	$ZnSO_4.7H_2O$	0.086	
	$CuSO_4.5H_2O$	0.00025	
F	Na_2 EDTA	0.373	20
	$FeSO_4.7H_20$	0.278	
G	Thiamine HCl	0.001	20
	Pyrodoxine	0.005	
	Nicotinic acid	0.005	
	Glycine	0.020	
	Myoinositol	100.00	
	Sucrose	30,000.00	
	Agar	6000-8000	

The observations were made at regular intervals of 24 hours and the development of callus was observed from the day of its formation. The data was statistically analyzed.

RESULTS AND DISCUSSIONS

In the present work earlier tissue culture works done on this plant were taken as base. For callus induction in MS auxins 2, 4-D and NAA were used in combination with KN and BAP in different proportions as 1:1, 4:1, 10:1 and 20:1. It was observed that when 2, 4 D was used with K maximum callus formation was observed in 10:1 (50%), and when with BAP then maximum callus in 4:1 (60%) (figure 1). Similarly when NAA was used with K then maximum callus formation was found in 10:1 (60%) and when with BAP then in 20:1 (50%) (table 2). (figure 1)

In the present investigation for redifferentiation BAP and KN were used in different concentrations like 0.2, 0.4, 0.5, 0.6, 0.8 and 1.0 ml/L for leaf primordial formation. Similarly for multiple shoot formation KN alone was used in 0.2, 0.3, 0.4 and 0.5 ml/L.

Maximum leaf primordia were found in higher concentrations of BAP, 60% in 0.6 ml/L and 50% in 0.8ml/L (Figure 2), while in case of KN leaf primordial were observed only in 0.4ml/L (Table 3). When shoot formation was attempted then shooting was only observed in 0.4ml/L K (50%) out of many other concentrations like 0.2, 0.3 and 0.4 ml/L (Table 4). When growth hormones NAA and BAP were used in combinations, shoots were observed only in 4:1 about 60% (Table 4) (Figure 3). For root formation both auxins and cytokinins were used in different combinations in 20:1. KN was found to give roots when used with 2,4 D as well as NAA 25% and 60% respectively. Where as BAP showed root formation only when used with NAA 50% (Table 5). (Figure 5).

Antonisamy and Manicham (1999) made attempts to conserve the *Withania* species through *in vitro* propagation and restore them in the natural habitats. Nodal segments and young shoot tips were used as explants for this. MS medium containing BAP were used in different combinations. For root induction shoots were put into the ½ MS medium supplemented with auxins.

Rani and Grover (1999) also used same growth hormones (2, 4-D; NAA with KN; BAP) in different combinations. They observed maximum callus (100%) formation from root and cotyledonary explants in 2, 4 D and KN. maximum shoot multiplication on MS containing BA. These shoots were rooted best (87%) on MS medium containing 2mg L^{-1}

Manickam *et al*. (2000) found maximum callus proliferation in MS with 2,4-D. Maximum shoot regeneration was achieved when callus was cultured on MS + BA + IAA. Rooting was observed on ½ MS + IBA. Furmanowa *et al*. (2001) produced *in vitro* plantlets from the shoot-tip of aseptically germinated seedlings. Kulkani *et al*. (2000) studied rapid micropropagation using nodes, internodes, hypocotyls and embryo explants. MS + benzyladenine (BA) or thidiazouron (TDZ) in various concentrations were used. Direct rooting was induced in leaf segments using an IBA dip treatment. The segments dipped in IBA formed roots along the midrib region of the abaxial surface when placed on MS medium containing no plant growth regulators.

CONCLUSION

In the present work *Withania somnifera* was subjected to callus induction and organ formation experiments. It was found that for callus formation NAA and KN combination was best in proportion 10:1. For leaf formation BAP was found to be most suited at 0.6 ml/L BAP. Similarly for shoot formation was observed in 0.5 ml/L Kinetin and in 4:1 ratio of NAA + BAP. When root formation was tried 20:1 NAA + K. was found to be most suitable.

Table 2. Data showing frequency of callus formation in different combinations

Medium	1:1	4:1	10:1	20:1
MS + 2,4-D + KN	20.0	13.3	50.0	25.0
MS + 2,4-D + BAP	20.0	60.0	20.0	0.0
MS + NAA + KN	40.0	13.3	60.0	60.0
MS + NAA + BAP	40.0	0.0	20.0	50.0

Figure 1. Pie chart showing average callus formation in different auxin : cytokinin ratio.

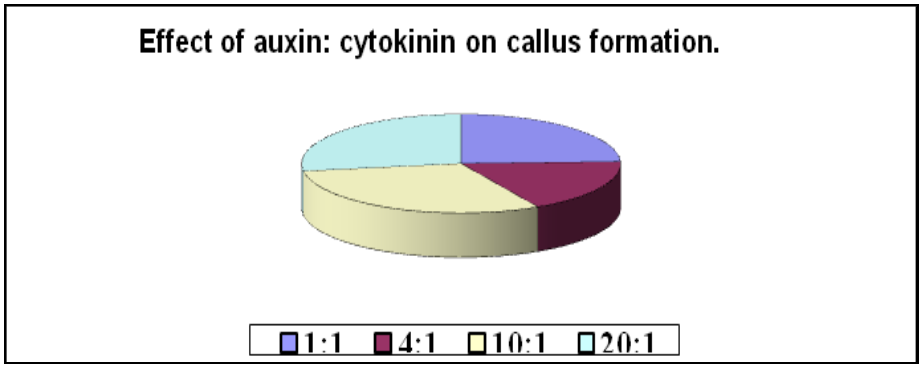

Table 3. Data showing frequency of leaf primordial formation in different concentrations of growth hormones.

Medium (ml/L)	0.2	0.4	0.5	0.6	0.8	1.0
MS + BAP	20	30	0	60	50	33.3
MS + KN	0	50	0	0	0	0

Table 4 . Data showing percentage shoot formation in different growth hormones

Medium (ml/L)	0.2	0.3	0.4	0.5
MS + KN	0	0	0	60
Medium (ratio)	1:1	2:1	3:1	4:1
MS + NAA + BAP	0	0	0	60

Table 4. Data showing percentage rooting in different growth hormones ratio

Medium	20:1
MS + 2,4-D + KN	25.0
MS + 2,4-D + BAP	0.0
MS + NAA + KN	60.0
MS + NAA + BAP	50.0

Figure 1. Callus formation in MS + 2, 4 D + BAP

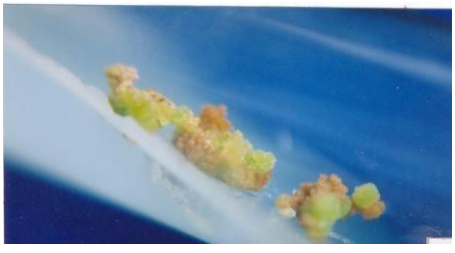

Figure 2. Leaf primordia formation in MS +(4:1) BAP (0.6ml/L)

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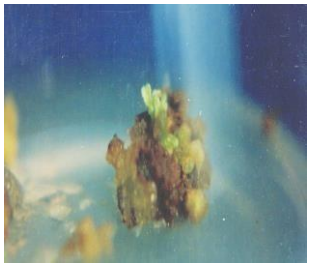

Figure 3. Shoot primordia in MS + NAA + BAP (4:1)

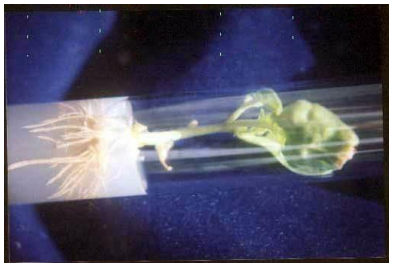

Figure 4. Root formation in MS + NAA + BAP and MS + NAA + KN

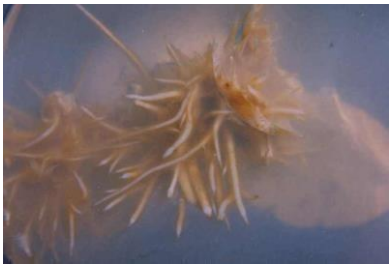

Figure 5.Root formation in MS+ NAA + BAP and MS + NAA + KN

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