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Study of Genetic Transformation of Medicinal Plants, *Withania Somnifera* (L.) Dunal By *Agrobacterium Tumefaciens* (MTCC-431)

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ABSTRACT
Genetic transformation of Withania somnifera (L.) Dunal was carried out using Agrobacterium tumefaciens strain MTCC-431. Explants such as leaf, root and seeds were used for genetic transformation. MS basal medium supplemented with different combination of hormones tried for regeneration of plants from different explants. Twenty four hours infection period for seedling was found suitable for genetic transformation. Soaking of seed prior to infection period of 72 hours produces 80% germination which was comparatively higher than that of observed at 24 and 48 hours. Different concentration of growth hormones GA_3 out of 2 mg/ml produces 60% germination in 7 days when seed are infected with 24 hours. Sucrose concentration of 15 mg respond better compared to 9 and 12 mg supplemented in MS growth medium. Enzymes localization on native PAGE for perioxidase, catalase, amylse and superoxide dismutase reveals appearance of new isozymes variants in infected seedling. HPLC analysis of gibbrellic acid treated and infected seedling versus infected seedling without showed different chromatogram. Present study was undertaken to standardize protocol for genetic transformation in a important medicinal plant will be useful for the enhancement of increased production of bioactive metabolites.
Key words: *Withania somnifera, Agrobacterium tumefaciens, HPLC, Isoenzyme*

INTRODUCTION

Withania somnifera popularly known as 'ASHWAGANDHA' is one of the major herbal components of geriatric tonics mentioned in Indian systems of medicine. It is a erect branching under shrub reaching about 150 cm in height, usually clothed with minutely stellate tomentum; leaves ovate up to 10 cm long, densely hairy beneath and sparsely above, flowers greenish or lurid yellow in axillary fascicles, bisexual, pedicel long, fruits globose berries which are orange coloured when mature, enclosed in a persistent calyx. The fleshy roots when dry are cylindrical, gradually tapering down with a brownish white surface and pure white inside when broken [1]. Majority of the constituents are withanolides (steroidal lactones with ergostane skeleton), glycowithanolides and alkaloids. These include withaferin A, withanolides G&D, sitoindosides and withasomnine [2-4].

In the traditional system of medicine Ayurveda, this plant is claimed to have potent aphrodisiac rejuvenative and life prolonging properties. It has general animating and regenerative qualities and is used among others for the treatment of nervous exhaustion, memory related conditions, insomnia, tiredness, potency issues, skin problems and coughing. It improves learning ability and memory capacity. The traditional use of 'Ashwagandha' was to increase energy, youthful vigour, endurance, strength, health, nurture the time elements of the body, increase vital fluids, muscle fat, blood, lymph, semen and cell production. It helps counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature aging emaciation, debility, convalescence and muscle tension. It helps invigorate the body by rejuvenating the reproductive organs, just as a tree is invigorated by feeding the roots [5-7].

In recent years market of plant products expand rapidly and this trend will continue in the 21st century because more and more people prefer natural products. Many of these products are difficult to synthesize chemically or difficult to produce in large amounts. Manipulation of the plant genome by introducing foreign genes has become a core tool in

plant biology. Plant transformation methods in use employ *Agrobacterium*, microprojectile bombardment, microinjection and electroporation of protoplasts. Among these, *Agrobacterium*-mediated plant transformation is the most extensively used method. It exploits the natural ability of *Agrobacterium* to transform plants to complete its own life cycle [8]. Plant transformation was initiated when *Agrobacterium tumefaciens*-mediated gene delivery was reported for the production of transgenic plants [9, 10]. It is now possible to transform a wide range of plants, including agronomically and horticulturally important crops, flowers and trees have been genetically modified using this method [11-12]. Most *Agrobacterium*-mediated transformations are carried out using *in vitro* tissue culture. Thus, transformation efficiency highly depends on the regeneration abilities of genotypes and explants. The production of transgenic rice plants by inoculating immature embryos with an *Agrobacterium tumefaciens* strain and proved the transformation by molecular and genetic analysis [13]. *A. tumefaciens*-mediated transformation of rice and maize were regenerated and characterized [14-15]. Transformation efficiency can be increased by the manipulation of either the plant or bacteria for enhancing competency of plant tissue and *in vitro* gene expression, respectively [16-17].

Agrobacterium tumefaciens-mediated transformation is generally used for genetic transformation of higher plants. *Agrobacterium*-mediated transformation has many advantages, such as low copy number of transgene, and stable inheritance of transgenes in a Mendelian fashion [10]. This study was carried out in order to obtain a fast, simple, reliable and high throughput method to genetically transform and allow us to establish an efficient secondary metabolic pathways identification and regulation platform of this famous medicinal plant.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIALS

Mature plant of *Withania somnifera* (Leaf, Root and Seed) was collected during June, 2010 to October, 2010 from the Indukaka Ipcowala College of Pharmacy, medicinal plant garden of New Vallabh Vidynagar, Gujarat India.

PLANT TISSUE CULTURE

The surface sterilization of different explants (Leaf, Root and Shoot) was carried out in 3 different steps. They were cleaned by running water for 2 h, wiped the superficial wax quality layer off with gauze, then surface sterilized by immersion in 75% alcohol for 30 s, followed by one rinse of 3 min in sterile distilled water, additionally immersed in 0.1% mercuric chloride ($HgCl_2$) solution for 1 min followed by three rinses of 5 min each in distilled water. The surface-sterilized different explants were blotted dry on sterile filter paper and planted on a semisolid agar (6.5 g l^{-1}) medium under the aseptic condition. The tube was then shifted to culture room with control facility of photoperiod (3000 lux) and temperature 25 ^{0} C. Murashige and Skoog (MS) basal medium supplemented with different combination of hormones (2,4-D, IBA, IAA, NAA) and sucrose (3%, 4% and 5%) were used for callus regeneration and different studies [18].

The viability of the seed was determined by soaking the seeds in distilled water for particular time intervals i.e. 24 h, 48h, 72 h. The non viable seeds were observed at the surface of water and were discarded immediately. The surface sterilization of seeds was carried out in 3 different steps. Seeds were immersed in lab wash containing beaker under running tap water for about 30 minutes. Then immersed into surface sterilization solution (0.1% $HgCl_2$) for approximately 30 sec to 1 min. Seeds were then washed with sterile distilled water 3 times and inoculated on MS medium [18] with different combination of sucrose (3%, 4% and 5%) with P^H 5.7 -5.8 and also used different combinations of hormones like GA_3, Kinetin and 2,4-D. The explants were inoculated to semisolid or liquid medium and transfered in the culture room at 25 ^{0}C temperature. Before the inoculation the Laminar Air Flow was subjected for UV light transmission for about 30 - 45 minutes. The inoculation of seeds was carried out under aseptic conditions under Horizontal Laminar Air Flow.

BACTERIAL CULTURE

Agrobacterium tumefaciens strain MTCC-431 strains was tested in a genetic transformation study for induction of callus and seeds from explants of *Withania somnifera*. The strain was obtained from Microbial Type Culture Collection (MTCC) Bank, Chandigarh as a freeze dried pure culture. The bacterial culture were revived by using MTCC specified selective growth medium and preserved as glycerol stocks. This is specialized strain of extremely good crown gall producer. *A. tumefaciens* belongs to the nopaline type. *A. tumefaciens* MTCC-431 strain was cultured and grown on nutrient broth medium. The cultures were maintained in the incubator at 29°C. Bacterial culture of the strain was initiated from fresh 1–2 day old streaked plates. The suspensions were initiated by transfer of single bacterial colony grown on NB agar media to NB liquid medium. These bacterial cultures were incubated in a shaker at 29°C at 120 rpm. Acetosyringone was incorporated in the liquid bacterial culture medium at concentrations ranging

from 0.05, 0.1, 0.6, 1, 10 to 25 mM after filter-sterilization. In another set of experiments, acetosyringone at concentrations 0.6, 1, 5, 10 and 25 mM was also used in the co-cultivation culture medium. The OD of the bacterial growth was taken after 12, 24, 48, 72 h and 96 h using spectrophotometer at 600 nm.

PHYTOCHEMISTRY

HPTLC ANALYSIS

For chemical profile analysis, dried root powder (1.0 g each) (wild and *in vitro* experiment material) was mixed with 3 ml methanol and kept it for 24 hrs after methanol is evaporated and collected the extracts. The methanolic extract was concentrated to 10 ml and used for HPTLC analysis (Camag system equipped with a sample applicator – Linomat-5, twin development chamber, TLC scanner-3 and integration software, documentation system Reprostar-3 with G5 digital camera) (Camag, Switzerland). HPTLC aluminium sheet pre-coated with silica gel 60 (1.05547 E Merck) was used as the adsorbent. Toluene: ethyl acetate: formic acid (5.0: 1.5: 0.5) were used as the mobile phase. The chromatographic development chamber was saturated with mobile phase for 10 min prior to placement of the plates. The plates were run up to 8 cm height and derivatized (10% H_2SO_4 in methanol). The derivative plates were heated at100°C for 4 min, bands were observed and scanned at 366 nm and photographs taken for record. *In vitro* root samples collected from culture room plants (n = 10) of infected and non infected of *Agrobacterium tumefaciens* were used in the study.

HPLC ANALYSIS

One gram dried root powder was extracted thrice in 50 ml methanol. Extracts were evaporated to dryness and reconstituted in methanol of HPLC grade according to the calibration curves. HPLC analysis was performed on a Perkin Elmer (Series, 200), USA system consisting of quaternary gradient system pump, RI (Range 1.00 to 1.75 RIV) detector, auto-sampler along with DGU 20A3 degasser and with Class VP software. Separations were achieved using column C-18, RP-18 and PI-Gel, 5 µm particle size and mobile phase of acetonitrile, and 1% acetic acid in water in the ratio of 60: 40 at a flow rate of 1 ml/min. The run the both sample with and without infection for analysis and comparative study. Each analysis was repeated twice.

RESULT AND DISCUSSION

In *Withania somnifera*, a preliminary experiment was performed to study *in vitro* induction of three types of explants (leaf, root, seeds) formed callus induction *in vitro*. These explants were inoculated on MS supplemented with various concentrations of either 2,4-D, KIN, IAA, IBA, NAA of different combinations. In leaf, MS medium supplemented with range of concentration of 2, 4-D (2, 2.5, 3 mg/l) with different combinations KIN (0.1, 0.2, 0.3, 0.4, 0.5, 1 mg/l), IAA (0.2, 0.3, 0.4, 1, 5, 10 mg/l), IBA (0.2, 0.3, 0.4 mg/l) and NAA (0.2, 0.3, 0.4 mg/l) have not responded. In root, MS medium supplemented with concentration of 2, 4-D with different combinations of 2, 4-D + KIN, 2, 4-D + IAA, 2, 4-D + IBA, 2, 4-D + NAA and IAA + KIN are inoculated. But the response of the explants survival was up to 30 days, and did not produce the callus induction.

In seed germination, MS medium supplemented with concentration of 2, 4 –D (2 mg/l) with combination of different concentrations of KIN (0.2, 0.5, 1 mg/l). The seeds are responding in concentrations 2, 4-D (2 mg/l) and KIN (0.5 and 1 mg/l) respond is 25% in this combination. Similar results have been obtained with other species results and are in conformity with some of the earlier findings in other plants of the same family, such as *Solanum laciriatum* [19], *Solanum sarrachoidea* [20], *Nicotiana tabaccum* [21] and *Solanum nigrum* [22]. Jayanti & Sharma,[23] reported in the same species with different combination of IBA, 2, 4-D & KIN comparative in result in 2, 4-D and KIN combination gives more response in seeds.

Induction of transformed roots

25 explants of leaf and seed were teretment with *A. tumefaciens* strain MTCC 431. In all cases, 25 explants were used as controls, leaf and seed explants infected with different time duration starting from 30 min, 1h, 2 h, 3 h, 4 h, 5 h, 6 h and 24 h. In contrast, leaf explants failed to responds to *A. tumefaciens* infection and became necrotic within 15 days of inoculation in both liquid and solid medium. In seed, MS medium supplemented in both liquid and solid medium, comparatively solid medium has shown more response compared to liquid medium(Table 1). The maximum result obtained from 24 h treatment of *A. tumefaciens* after 10 days showing 60% response. Similar type of result observed by Mohammed *et al* (2009) [24] in *Corrchorus olitorius*.

Table -1: Effect of time duration in seed germination for genetic transformation in *W. somnifera*

Infection Duration	Liquid Medium		Solid Medium	
	No. of Explants	% respond	No. of Explants	% respond
30 min	25	05	25	10
1h	25	15	25	25
2h	25	20	25	30
3h	25	20	25	30
4h	2 5	25	2 5	40
5h	25	20	25	45
6h	25	20	25	45
24h	25	45	25	60

Water soaking treatment

To check the effect of water soaking of seeds on seed germination. The seed are soaked in water for different time duration of 24, 48, 72 hours and then subjected to *A .tumefaciens* infection for 24 h. After 10 days result, it was observed that infection of *A. tumefaciens* was maximum in water soaking treatment 72hrs seeds. The 80% response within 7 days was achieved compared without infection here seed germination occur at very low frequency (Table 2). Hosakatte *et al*, [30] reported seed germination in the same plant species but against *A .rhizogenes* but without water soaking treatment. Based open the result are water soaking treatment are more favorable for this plants.

Table -2: Effect of water soaking of seeds on seed germination of *W. Somnifera.*

CONSTITUENTS	Water Soaking Time Duration	No. of Seeds	Infection Duration (in hr)	After infection No. of Days	With out Infection % of Germination	With infection % of Germination
MS MEDIUM	24	100	24 h	10	10	60
	48	100	24 h	9	10	70
	72	100	24 h	7	10	80

Effect of different concentration of GA_3 supplementation (0.5, 1, 2 mg/l) with infection of *A. tumefaciens*.

Germinated seed grown in MS basal medium supplemented with different concentration of GA_3 such as 0.25, 0.5, 1, 2 and 3 mg/l. In each combination 25 seeds were inoculated. Out of these, maximum response was observed in the 2 mg/l within 7 days. The rest of the combination responded very slow and taken more time for the germination. The concentration of 2 mg/l is good for *W. somnifera* (Table 3). Anjali *et al*, [25] also reported seed germination in the same species by using the BA & TDZ hormones. The germinated seed are infected with *A. tumefaciens,* maximum response was observed in the 2 mg/l GA_3 containing medium within 7 days. The rest of the concentration also gives response but less compared to 2 mg/l and takes more time for germination. *Agrobacterium rhizogenes* mediated hairy root induction was also reported in two medicinally important same members of family Solanaceae [26].

Table -3: Effect of different concentration of GA3 supplementation with infection of *Agrobacterium tumefaciens* on seeds of *Withania somnifera*

CONSTITUENTS	No. of seeds inoculated	Infection Duration (Time in hr)	No. of days taken for Germination	% of seeds Germination
0.5 mg/l	200	24 h	7-8 days	55
1 mg/l	200	24 h	8-9 days	55
2 mg/l	200	24 h	7 days	65

Effect of different sucrose concentration on seed germination with MS medium

The different sucrose concentration like 9 gm/ l, 12 gm/ l and 15 gm/ l were taken in MS medium and seed infected with *Agrobacteruim tumefaciens* was treated for 24 hr. The maximum response was observed in the 15 gm/100 ml within 9 days as compared to other combinations (Table 4). Similar type of observation was reported by Asha and Nutan, [27] in green hairy root cultures of *Solanum khasianum*, new route to *in vitro* Solasodine production. Sugars are involved in the differentiation of xylem & phloem, elements in cultured cells [28]. We also observed growth improvement at 15 mg/l sucrose concentration.

Table -4: Effect of different sucrose concentration on seed germination of *Withania somnifera*

CONSTITUENTS	Sucrose Conc. (g/1it)	No. of seeds inoculated	Infection Duration (Time in hr)	No of Days taken for Germination	% of Germination
MS Medium	9	100	24	8	50
MS Medium	12	100	24	8	54
MS Medium	15	100	24	9	62

Enzyme localization

Enzymes are very important for the production of secondary metabolites. Some enzymes like peroxidase, catalase and amylase are involved in such process. After infection of *A. tumefaciens* in seed, the 15 days old seedling was used to study the localization of enzymes through native gel electrophoresis. The catalase enzyme after infection of *A.tumefaciens* in seeds produce one new bands of isoenzyme compared to without infection of seeds. The peroxidase enzyme band was compared with control and found that it was thicker and one new band was also observed. Similarly amylase enzyme was also compared. In this enzyme, one band of isoenzyme is produce. The superoxidase dismutase (SOD) enzyme plays a key role in the organogenesis and morphogenesis. In SOD enzyme, also there is an occurrence of new band compare to control. Isoenzymes have been widely used in studies on the evolution of plants. The pattern and band intensities can vary with the tissue used and their stages of development [29]. These enzymes may play an important role in the production of metabolites.

HPLC and HPTLC Analysis

Methnolic extract of 15 days old infected seeds were used for HPLC and HPTLC analysis to compare production of secondary metabolites in normal and treated plants. Study of the sample of infected seeds with our result on the present study indicate that some of the band were newly found in the hormone with infection of *A .tumefaciens* compared to without hormones treatment. The 2D graph and spectral analysis comparision indicates that *A. tumefaciens* infected plant produce new peak in TLC plate indicating presence of new metabolite produce after treatment. The different spectral study at 254nm and 365nm were carried out for the detection of presence or absence of bands. The induction, by *A. tumefaciens* may produce different chemotypes in *W. Somnifera.*

The result of transformed roots and non-transformed root cultures using HPLC analysis was depicted in Figure 1A, 1B and 1C. The UV spectrum and retention time of the peak obtained was used to identify the corresponding peak of wild plant root extracts. The methanolic extract peak also compared between the seeds infected with *A. tumefaciens* on without hormones and with hormones (Figures 1A, 1B and 1C). Hormones treatment with infection shows one more peak which indicates that production of new secondary metabolites after infection. Hosakatt *et al*., (2008) also studied the withanolid content in transformed and non-transformed cultured roots in *A. rhizogenes* in *W. sommnifera* [30].

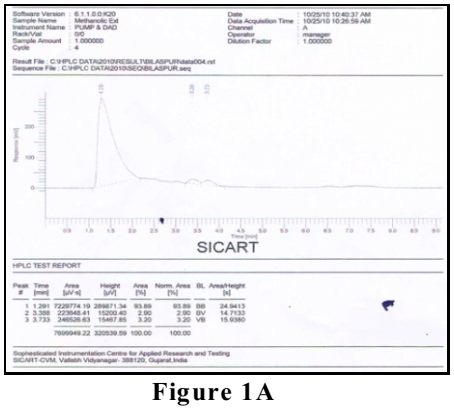

Figure 1A

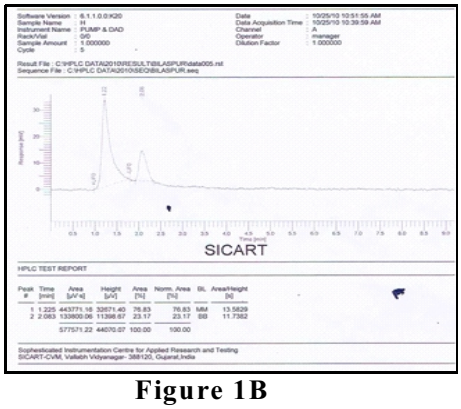

Figure 1B

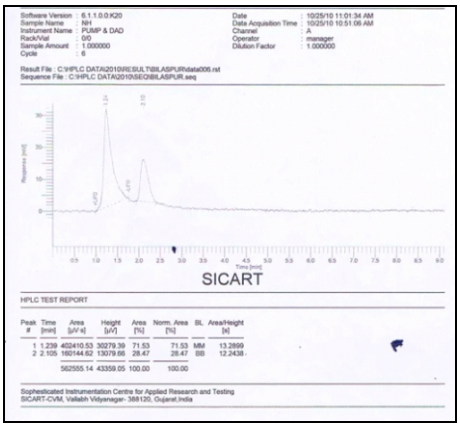

Figure 1C

Figure-1. HPTLC analysis of *Witania somnifera* 1A. Methanolic extract 1B. Hormones with infection 1C. Without hormone infection

CONCLUSION

Present study was undertaken to standardize protocol for genetic transformation in an important medicinal plant, *Withania somnifera* which in turn will be useful for the enhancement of active principle. Different parameters such as infection treatment by *A. tumifaciens*, time duration, gibbrellic acid and sucrose concentrations optimized in this study could be of great importance as far as increased production of bioactive metabolites is concerned. Additional studies are required to produce bulk amount of bioactive metabolites of commercial importance.

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REFERENCES

[1]. Pandey, C.N., Raval, B.R., Mali, S. and Salvi, H (2005) Medicinal plants of Gujarat, Gujarat Education and Research Foundation, Gandhinagar.pp-1-5.
[2]. Ghosal, S., Kaur, R and Bhattacharya, S (1988) Chemistry and bioactivity of sitoindosides IX and X. *Planta Med,* 54:561.
[3]. Glotter, E (1991) Withanolides and related ergostane-type steroids. *Nat Prod Rep*, 8: 415–440.
[4] Matsuda, H., Murakami, T., Kishi, A and Yoshikawa, M (2001) Structures of withanosides I, II, III, IV, V, VI, and VII, new withanolide glycosides, from the roots of Indian *Withania somnifera* and inhibitory activity for tachyphylaxis to clonidine in isolated guinea-pig ileum. *Bioorg Med Chem,* 9:1499–1507.
[5]. Nadkarni, K.M and Nadkarni, A.K (1976) Indian Materia Medica. VII.3rd ed. Mumbai: M/S Popular Prakasan Pvt.Ltd.
[6]. Mishra, L.C., Singh, B.B and Simon, D (2000) Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha): a review. *Altern. Med. Rev*, 5(4):334–346.
[7]. Kapoor, L.D (2001) Handbook of Ayurvedic Medicinal Plants; CRC Press: London, UK,pp-337-338.
[8]. Otoni, W.C., Picoli, E.A., Costa, M.G., Nogueira, F.T and Zerbini, F.M (2003) Transgenic Tomato; in Plant genetic engineering-a multivolume series: 1 vol. 5 (eds), Houston: Sci-Tech. Pub. Co).pp- 41–131.
[9]. Horsch, R.B., Fry, J.E., Hoffmann, N., Eicholz, D., Rogers, S.G and Fraley, R.T (1985) A simple and general method for transferring genes into plants. *Science*, 227: 1229-1231.
[10]. Gelvin, S.B (2003) Agrobacterium-mediated transfor- mation the biology behind the "Gene Jocking" tool. *Microbiol Mol Biol Rev*, 67:16-37.
[11]. Ko, T and Korban, S (2004) Enhancing the frequency of somatic embryogenesis following Agro bacterium-mediated transformation of immature cotyledons of soybean (Glycine max (L.) Merrill). *In Vitro Cell Dev Bio-Plant*, 40:552-558.
[12]. Lopez, S.J., Kumar, R.R., Pius, P.K and Muraleedharan, N (2004) *Agrobacterium tumefaciens* mediated genetic transformation in tea (Camellia sinensis (L.) O. Kuntze). *Plant Mol Biol Rep*, 22: 201a-201j.
[13]. Chan, M.T., Chang, H.H., Ho, S.L., Tong, W.F and Yu, S.M (1993) Agrobacterium-mediated production of transgenic rice plants expressing a chimeric a-amylase promoter P-glucuronidase gene. *Plant Mo1 Biol*, 22: 491-506.
[14]. Lin, Y.J and Zhang, Q (2005) Optimizing the tissue culture conditions for high efficiency transformation of Indica rice. *Plant Cell Rep,* 23: 540-547.

[15]. Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T and Kumashiro, T (1996) High efficiency transform- ation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens. Nature*, 14:745-750.

[16]. Mondal, T.K., Bhattacharya. A., Ahuja, P.S and Chand, P.K (2001) Transgenic tea (*Camellia sinensis* (L.) O. Kuntze cv. Kangra Jat) plants obtained by Agrobacterium-mediated transformation of somatic embryo. *Plant Cell Rep,* 20:712-720.

[17]. Chakrabarty, R., Viswakarma, N., Bhat, S.R., Kirti, P.B., Singh, B.D and Chopra, V.L (2002) Agrobacterium-mediated transformation of cauliflower: optimiza- tion of protocol and development of Bt-transgenic cauliflower. *Journal of Biosciences,* 27: 495-502.

[18]. Murashige, T and Skoog, F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*, 15:473–497.

[19]. Chandle, S.F., Dodds, J.H and Henshaw, G (1982) Factors affecting adventitious shoot for mention in solanum laciniatum lit, callus cultures, 5th meeting on plant tissue culture. Pp-133-134.

[20]. Banerjee, S., Schwemmin, A.M and Schwemmin (1985). *In vitro* propagation of Solanum sarrachoides Sendt. *J. Plant physiology*, 19:461-166.

[21]. Rathore, K.S and Goldsworthy, A (1985) Electrical control of shoot regeneration in plant tissue cultures. *Biotechnology,* 3:1101-1109.

[22]. Shahzad, A., Hasan, H and Siddiqui, S.A (1999) Callus induction and regeneration in solanum nigrum. *In vitro phytomorphology*, 49: 215-220.

[23]. Jayanti, S and Sharma, A.K (1991) Micropropagation of *Withania somnifera* from germinating seeds and shoot tips. *Plant Cell, Tissue and Organ Culture*, 26:71-73.

[24]. Mahammad Saiful Islam., Kazi md. kAmrul Huda., Firoz Mahmud., Sufara Akhter Banu and myeomg-Hyeonwang (2009) Regeneration and genetic transformation of Tossa Jute (*Corchorus olitorius* L.). *Australin Journal of Crop Science*, 3(5): 287-293.

[25]. Anjali, A. K., Thangane, S.R. and Krishnamoorthy, K.V. (2000). Direct shoot regeneration from node, internodes, hypocotyl and embryo explants of *Withania somnifera*. *Plant Cell Tiss. & Org. Cult*. 62: 203-209.

[26]. Pankaj, K., Pawar, Vijay and Maheshwari, L (2004) Agrobacterium rhizogenes mediated hairy root induction in two medicinally important members of family Solanaceae. *Indian journal of Biotechnology,* 3:414-417.

[27]. Asha, Jacob., Nutan and Malpathak.(1991) Green hairy root cultures of Solanum Khasianum. Clarke-a new route to in vitro solasodine production. *Current sciences,* 87(15):1442-1447.

[28]. George, E.F and Shrington, P.D (1984) In plant propagation by tissue culture – Hand Book and Directory of commercial Laboratories, Exegetics Ltd, Eversley, England.

[29]. Alfenas, A., Peter, C., Brune, W and Passador, G.C (1991) Electroforese de protinase isoenzymes de fungus e essencius florestais,Viscosa, 11-24.

[30]. Hosakatte, Niranjana Murthy., Eun, Joo Hahn and Kee, Yoeup Paek (2008) Adventitious oots and Secondary Metabolism. *Chinese Journal of Biotechnology* 24(5):711-716.

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