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Research Article

COMPARATIVE EVALUATION OF *IN VITRO* REGENERATION POTENTIAL OF SEEDS OF *W. SOMNIFERA* AND *W. COAGULANS*

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ABSTRACT

***Withania somnifera* and *Withania coagulans* have been well known to possess tremendous medicinal potential. Owing to this both the plants find their extensive utilization in traditional as well as modern medicine system. Unrestricted collection from natural stands, destruction of natural habitat and low propagation rate in nature has rendered both the plants endangered. Seeds of both the species exhibits poor germination rate in nature. In the present study, effect of HCl and H_2SO_4 on seed germination was studied. Culture of germinated seeds onto MS medium fortified with different concentration of NAA and BAP resulted in callusing and regeneration of multiple shoots. Well elongated shoots were rooted onto medium containing IBA. Half strength MS medium was far superior for root induction as compared to full strength for both the species. A maximum of 94.6% cultures of *W. somnifera* and 84.6% cultures of *W. coagulans* developed *in vitro* roots onto ½MS+10µM IBA and ½MS+20µM IBA respectively. About 75.6% plants of *W. somnifera* and 64.9% plants of *W. coagulans* survived during the process of hardening and transplantation.**

Keywords: Germination, HCl, H_2SO_4, *in vitro* culture, Napthalene acetic acid, Benzyl amino purine, Indole acetic acid.

INTRODUCTION

Among the 23 known species of Genus *Withania*, *Withania somnifera* and *Withania coagulans* are two species specifically known for their economic and medicinal value (Mirjalili *et al.*, 2009). *W. somnifera*, commonly known as Ashwagandha or Indian Ginseng is a cosmopolitan plant, found to be distributed in drier parts of subtropical India, Pakistan, Afghanistan, Egypt, Morocco, Spain, Canary Island, Eastern Africa Congo, Madagascar and South Africa (Wealth of India, 1982., Farooqui and Sreeramu, 2004., Sharma *et al.*, 2014a). In India, it is native to drier parts (Logesh *et al.*, 2010.,

Baba *et al.,* 2013) and cultivated in selected areas like Neemuch and Jawad tehsils of Mandsaur district of Madhya Pradesh, Mysore in Karnataka, Punjab and Rajasthan (Farooqui and Sreeramu, 2004). *W. somnifera* is among, one of the most important medicinal herb of Ayurveda and many preparations containing the herb have been commercialized (Tripathi *et al.*,1996., Sukh Dev 2006., Singh *et al.*,2010).

The plant is known to exhibit many medicinal properties such as anti-inflammatory, anti-stress, anti-tumor, antioxidant, antineoplastic effects, rejuvenating, immunomodulatory activity, cardioprotective activity, hypothyroid activity along with antimicrobial activity against several bacteria and fungi (Anbalagan and Siddique 1984, Bhattacharya *et al.,* 1997., Mishra *et al.,* 2000, Winter, 2006., Bone, 2009., Ojha and Arya, 2009., Verma and Kumar 2011, Sharma *et al.,* 2014a). *W. somnifera* possesses slow propagation rate in nature. Sen and Sharma (1991) and Kumar *et al.,* (2013) have reported absence of vegetative propagation in *W. somnifera*. It is commonly propagated by seeds but germination in *W. somnifera* is poor (Vakeswaran and Krishnasamy, 2003). Karnik (1978) and Kumar *et al.,* (2011) reported presence of certain inhibitors in fruits of *W. somnifera* which adversely affect germination. Decrease in seed viability with time has also been a major concern for declining population of the plant. (Rani and Grover 1999., Silva and Sreenath, 2009).

Withania coagulans, commonly known as Indian rennet or Doda paneer is an important endangered medicinal plant of immense medicinal value. The plant is found to be distributed throughout Pakistan, North-West India and Afghanistan (Mathur and Agarwal, 2011). *W. coagulans* is used in treatment of various ailments and diseases such as nervous exhaustion, disability, insomnia, wasting diseases, impotence, dyspepsia, flatulent colic and other intestinal infections (Dymock *et al*., 1972., The Wealth of India, 1969). Berries of plants are used as blood purifiers and flowers have been reported to be antidiabetic (Bown, 1995). The twigs of plant are chewed for cleaning of teeth and smoke of plant is inhaled for relief in tooth ache (Mathur and Agarwal, 2011). Beside these medicinal properties, plant is known to possess various pharmaceutical activities like anti-microbial, anti-inflammatory, anti-tumor, hepatoprotective, anti-hyperglycemic, cardiovascular, immune suppressive, free radical scavenging and CNS depressant activities of the plant (Jaiswal *et al.,* 2009., Maurya and Akansha, 2010., Mathur *et al.,* 2011., Pezeshki *et al.,* 2011., Khodaei *et al.,* 2012.

Destruction of natural habitat, low propagation rate in nature and over exploitation from natural resources for medicinal and traditional purposes have rendered the plants endangered (Govil *et al.,* 1993., Vitali *et al.,* 1996, Manickam *et al.,* 2000 and Sharma *et al.,* 2011). Beside this, reproductive failure due to dioceious nature of *W. coagulans* is chief problem in its mass propagation (Jain *et al.,* 2009).

Therefore, tissue culture studies become significant as well as essential for mass propagation and conservation of both the species. Siddique *et al.* (2004)., Sangwan *et al.* (2007)., Sivanesan and Murugesan, (2008)., Shukla *et al.* (2010)., Sharma *et al.* (2014b) have reported tissue culture studies of *W. somnifera* through

nodal segment culture. Logesh *et al.* (2010) and Arumugam and Gopinath (2011) utilized leaves for their respective studies. However, there are very few studies conducted which have utilized seeds as explants (Kulkarni *et al.,* 1996., Kanungo and Sahoo, 2011). Not many reports are available with respect to tissue culture studies of *W. coagulans*. Jain *et al*. (2009) and Valizadeh and Valizadeh (2011) have been the prominent workers to have conducted micropropagation studies.

Hence, present study was conducted to develop an easy, efficient and simple method for enhancing germination rate of both the species along with mass propagation through *in vitro* culture of seeds.

MATERIALS AND METHODS

Fruits of *W. somnifera* and *W. coagulans* (Doda paneer) were procured from local market and nurseries. Fruits were thoroughly washed under running tap water for one hour to separate out the seeds. Following the isolation, seeds were given different pretreatment for enhancement of germination rate. Seeds of both the species were soaked in sterile distilled water for 24-72 hours before incubation for germination. In separate experiments seeds were treated with different concentrations of HCl (5-20%) and H_2SO_4 (5-20%); for varying durations of time (10-20 minutes). Both the chemicals were of grade AR (analytical reagent) procured from SDFCL (sdFine Chemicals Ltd. Mumbai). Following the treatment, seeds were thoroughly washed 2-3 times with sterile distilled water to remove all traces of acid. Seeds rinsed with distilled water were utilized as control. After the treatment, seeds were aseptically transferred to sterile Petri plates containing moistened cotton or filter paper. In a separate experimental setup treated seeds were transferred to pots containing sterile soil and sand in 1:1 ratio. In both the experimental setups, seeds were incubated at room temperature. Petri plates and pots were appropriately labeled and regularly monitored for germination of seed. Prior to inoculation germinated seeds were surface sterilized by treating them with 0.2% v/v solution of Tween-20 and kept under running tap water for about 10 minutes. Seedlings were then rinsed with 90% ethyl alcohol for 10-15 seconds and finally surface sterilized with 0.1% $HgCl_2$ for 2 minutes and then repeatedly washed with sterile distilled water to remove all traces of $HgCl_2$. Seedlings were dried using sterile tissue paper and inoculated onto prepared MS (Murashige and Skoog's, 1962) media. The germinated seedlings were transferred to MS basal medium as well as medium containing different plant growth regulators 2,4-D, NAA and Kn in various concentration (2-12µM) for further growth and development. Callus containing shoot buds were aseptically excised and transferred to fresh MS medium containing same hormone, BAP (5-12µM) for further proliferation and elongation. Well elongated *in vitro* regenerated shoots were transferred to freshly prepared rooting medium containing 1BA (2-20µM). Both full and half strength MS medium were utilized for rooting. Completely developed plants were removed from culture vessel under aseptic conditions and thoroughly washed with sterile water to remove all traces of media. Plants were then transferred to plastic pots containing sterile soil and sand (2:1). Pots were covered with transparent polybags and kept in culture room and were irrigated with half strength MS nutrient solution as per requirement.

After emergence of new leaves, plants were transferred to green house. After 20-25 days of growth in green house plants were finally transferred to natural soil. All the cultures were regularly monitored for growth and development. Germination of seeds was observed up to 30 days from day of incubation. Every experiment was repeated at least twice and each experimental setup consisted of minimum of 20 cultures. Data presented represents mean value followed by standard error.

RESULTS AND DISCUSSION

Seed germination studies

In control, a maximum of 21.4% seeds of *W. somnifera* germinated (Fig. 2A) while germination in *W. coagulans* was only 1-3% (Fig. 2B) after four weeks of incubation. However, when the seeds were soaked in sterile distilled water for about 72 hours before transferring them to Petri plates, 48.6% seeds of *W. somnifera* and 22.0% seeds of *W. coagulans* germinated after 28 days of incubation. Similar results have also been reported by Niyaz and Siddiqui (2014) who also achieved increased germination rate by soaking seeds of *W. somnifera* in water for 24-48 hours.

Effect of HCl on germination rate

Significant enhancement in germination rate was obtained when seeds of both the species were pretreated with different concentration of HCl. When seeds were pretreated with 5% HCl for 10 minutes, about 52.4% seeds of *W. somnifera* and 38.5% seeds of *W. coagulans* germinated after 3-4 weeks of incubation. When concentration of HCl was increased upto 10% for 10 minutes, germination percentage obtained was 68.8 and 48.6 for *W. somnifera* and *W. coagulans* respectively. Keeping the concentration of HCl same (10%) and on increasing the duration of treatment to 20 minutes, about 81.5% seeds of *W. somnifera* (Fig.3A) and 64.4 % seeds of *W. coagulans* (Fig.3D) germinated within 28 days of treatment. On further increasing the concentration of HCl to 20%; rate of germination was accordingly enhanced. Seeds treated with 20% HCl for 10 minutes resulted in germination of 76.4% seeds of *W. somnifera* and 63.2% seeds of *W. coagulans* (Fig.1). When the duration of treatment was increased to 20 minutes germination percentage of seeds of *W. somnifera* and *W. coagulans* increased to 80.2% and 72.6 % respectively within 28 days of incubation (Fig. 4A,B). Pandey *et al.*(2013) in their study also soaked the seeds of *W. somnifera* in 0.1N HCl overnight followed by other treatment to enhance germination rate. In the same study they also reported that excision of seeds also resulted in enhanced germination.

Effect of H_2SO_4 on germination rate

Treatment of *W. somnifera* and *W. coagulans* seeds with H_2SO_4 also resulted in enhancement of germination rate. When seeds were treated with 5% H_2SO_4 for 10 minutes about 28.4% seeds of *W. somnifera* germinated after 7 days of incubation. The germination percentage was enhanced upto 52.6% after 28 days of incubation onto same treatment. Contrastingly, only 28.2% seeds of *W. coagulans* germinated after 28 days of incubation in same treatment. When the duration of treatment was increased upto 20 minutes to the same concentration of H_2SO_4, 68.8% seeds of *W. somnifera* and 42.4% seeds of *W. coagulans* germinated after 28 days of treatment (Fig. 1). When the concentration of H_2SO_4 was gradually increased to 10%, germination percentage was accordingly enhanced. About 66.3%

and 32.6% seeds of *W. somnifera* and *W. coagulans* respectively germinated after 10% H_2SO_4 treatment for 10 minutes (Fig.3C,3D). Germination rate of *W. somnifera* and *W. coagulans* was enhanced to 80.9% and 60.4% respectively when the duration of treatment was enhanced to 20 minutes. On further increasing the concentration of H_2SO_4 to 20% respective enhancement in germination rate was observed. A maximum of 82.5% and 78.4% seeds of *W. somnifera* and *W. coagulans* respectively germinated when treated with 20% H_2SO_4 for 20 minutes (Fig.4C,4D). Comparatively seeds of *W. somnifera* exhibited a better response to both HCl and H_2SO_4 as compared to that of *W. coagulans*. No significant difference in germination rate was achieved whether the treated seeds were germinated in pots containing sterile soil (Fig. 5A-D), however soil germinated seeds did not exhibit good survival rate and only 48.6% seeds of *W. somnifera* and 2% seeds of *W. coagulans* survived when germinated in soil unless transferred to culture medium. Extremely poor survival rate of germinated seeds of *W. coagulans* and moderate survival rate of germinated seeds of *W. somnifera* supports utilization of plant tissue culture technique for conservation of the species.

Culture of germinated seeds

When 8-10 days old germinated seedlings of both *W. somnifera* and *W. coagulans* were transferred to basal medium, initially slow growth was obtained, but after 2-3 weeks rapid growth was observed resulting in complete plant regeneration.

In vitro culture of seedlings of *W. somnifera* and *W. coagulans* onto MS +BAP (2-12µM), resulted in callus development among all the cultures within 1-2 weeks of culture. Callus developed from culture of seedlings of *W. somnifera* was followed by regeneration of shoot buds from callus onto all concentrations of BAP (Fig.9A, 9B). MS+12µM BAP yielded best results as about 9.8±0.5 shoots/culture with a maximum shoot length of 5.6cm. Callus obtained was fragile and green. Morphogenic results obtained from culture of seedling of *W. somnifera* were uniform on all concentrations of BAP. However, seedling of *W. coagulans* exhibited different morphogenic response onto lower and high concentration of BAP. On to lower concentration of BAP (2-5µM), predominantly callus development was achieved with 66.6% cultures exhibiting regeneration of shoot buds from regenerated callus onto MS+5µM BAP (Table 1). With higher concentration of BAP (8-12 µM), callus development as well as regeneration of shoot buds from callus was achieved in all the cultures (Fig. 9C). On an average 6.4±1.2 shoot buds regenerated from callus with a maximum shoot length of 6.1cm. *In vitro* culture of seedlings of both the species onto MS medium supplemented with different concentrations of NAA (2-12µM) resulted in callus formation irrespective of concentration of NAA. All the cultures developed callus within 8-10 days of incubation. Callus obtained from seedlings of *W. somnifera* was yellowish green and crystalline (Fig. 6A, 6B). Callus obtained from seedlings of *W. coagulans* onto lower concentration of NAA (2-5µM) resulted in green and compact callus development (Fig.7A-C). However, onto higher concentration of NAA (8-12µM) initial callus development (Fig. 8A) was followed by formation of hairy roots onto higher concentration of NAA (Fig. 8B).

However, shoot bud regeneration was not achieved from regenerated callus from culture of either species onto NAA enriched medium. In contrast to the results obtained in the present study Sen and Sharma (1991) reported regeneration of multiple shoots from seedlings without formation of intervening callus onto medium containing IBA and 2,4-D. Similarly, Kulkarni *et al.*(1996 and 1997) also achieved regeneration from seedlings into MS medium enriched with TDZ and BAP.

Proliferation and elongation of shoots

Subculture of callus containing shoot buds onto BAP supplemented medium resulted in extensive proliferation of shoots. A maximum of 12 shoots with an average of 09±1.2 shoots per culture were obtained onto MS+12µM BAP. From results obtained in the present study it can be stated that after enhancing the germination rate seedlings can be successfully utilized as explants for tissue culture studies.

***In vitro* rooting**

When regenerated shoots of *W. somnifera* and *W. coagulans* were transferred to IBA enriched MS medium for *in vitro* root formation following mentioned results were obtained. Regenerated shoots of *W. somnifera* developed roots onto full as well as half strength MS medium containing IBA (5-20µM). About 42.5% cultures developed roots onto full strength MS medium containing 20 µM IBA. Whereas percentage of cultures developing roots was enhanced to 90% when strength of medium was reduced to half with same concentration of IBA (Table 2). An average of 11.2±0.4 roots per culture were obtained onto ½ MS+20µM IBA (Fig. 10A). Maximum root length obtained onto this medium was 8.2cm. Hence, in the present study ½ MS+IBA were found to be far superior as compared to full strength MS medium for *in vitro* rooting of *W. somnifera*. Similar results have also been reported by Sivanesan (2007), who also supported ½ MS+IBA ideal media composition for rooting. However, results obtained in respective studies of Sharma *et al.*(2010)., Fatima and Anis, (2011)., Kumar *et al.*(2011) reported full strength MS medium containing IBA to be sufficiently effective enough for *in vitro* induction of roots.

On the contrary, regenerated shoots of *W. coagulans* failed to develop roots onto full strength MS medium irrespective of concentration of IBA. However, when strength of medium was reduced to half, rooting was achieved. About 84.6% cultures developed roots onto ½ MS +20µM IBA with an average of 12.05±0.9 roots per culture, maximum root length was 10.6 cm. (Fig.10B). Roots obtained were thick and white. Similar to the findings of present study, Valizadeh and Valizadeh, (2011) also achieved *in vitro* rooting onto ½ MS+IBA whereas Jain *et al.*(2009), reported IBA to be suitable enough for *in vitro* rooting in *W. coagulans.* About 75.6% plants of *W. somnifera* and 64.9% plants of *W. coagulans* survived during the process of hardening and transplantation.

Conclusion

The present study provides an efficient and easy protocol for mass propagation as well as conservation of *W. somnifera* and *W. coagulans*. The protocol can be effectively utilized for rehabilitation of plants with specific reference to *W. coagulans*. Also, the protocol can prove useful to assess the biochemical and molecular aspects

responsible for poor germination rate and poor survival of germinated seeds under natural conditions.

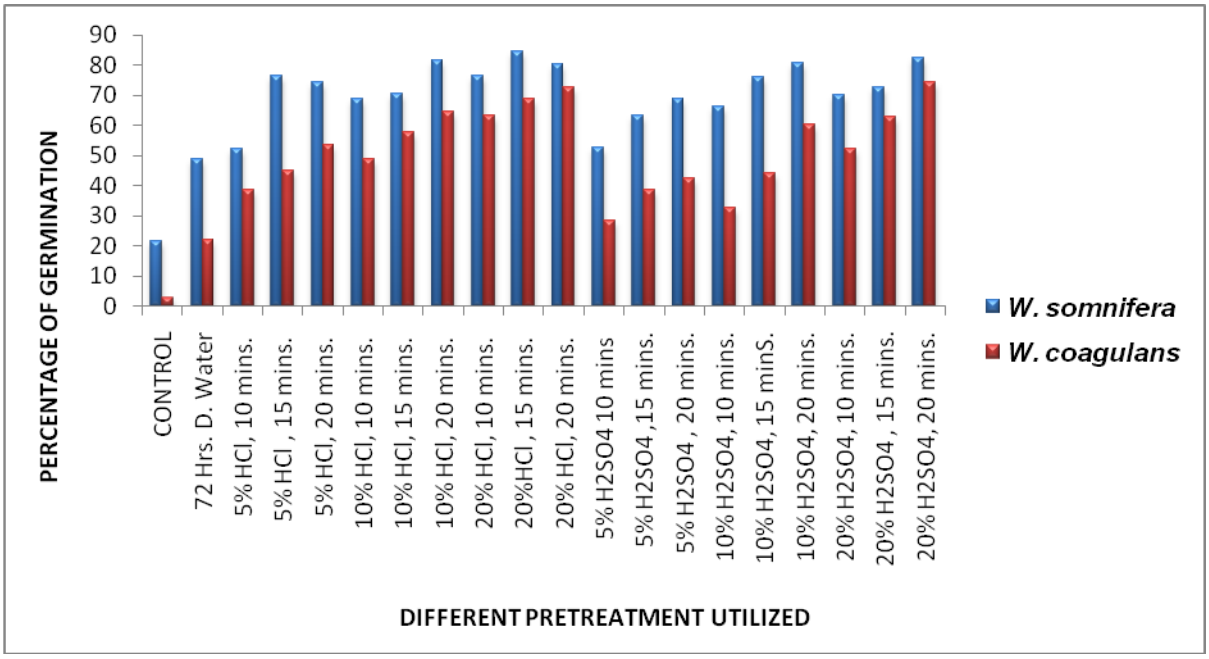

Fig. 1: Effect of HCl and H_2SO_4 on germination rate of *W. somnifera* and *W. coagulans*

Table 1: Effect of BAP on seedling culture of *W. somnifera* and *W. coagulans*

BAP (µM)	Degree of Callusing		% of culture exhibiting shoot bud regeneration		Average number of shootbuds		Maximum shoot length (cm)	
	WS	*WC*	*WS*	*WC*	*WS*	*WC*	*WS*	*WC*
2	+++	++	100	48.4	4.3±0.6	3.2±0.4	4.0	2.5
5	+++	++	100	66.6	6.1±1.2	3.0±0.2	4.2	2.7
8	+++	+++	100	100	8.4±0.8	4.8±1.0	6.5	5.8
10	+++	+++	100	100	8.0±0.2	6.2±0.8	6.3	6.3
12	+++	+++	100	100	9.8±0.5	6.4±1.2	5.6	6.1

WS (*Withania somnifera*), WC (*Withania coagulans*), +, Poor callusing; ++, Moderate callusing; +++, Good callusing; ++++, Extensive callusing

Table 2: Effect of IBA on *in vitro* rooting

Media Composition	*W. somnifera*			*W. coagulans*		
	% culture developing roots	Avg. No. of roots	Max. root length(cm)	% culture developing roots	Avg. No. of roots	Max. root length(cm)
MS+ 5 µM IBA	24.2	4.0±0.4	4.2	-	-	-
MS+ 10 µM IBA	36.4	3.9±0.2	5.6	-	-	-
MS+ 20 µM IBA	42.5	4.6±0.6	5.4	-	-	-
½ MS+ 5 µM IBA	78.8	8.2±0.5	7.9	67.4	09.02±0.7	5.6
½MS+ 10µM IBA	94.6	11.0±1.2	8.7	82.4	14.06±1.2	10.2
½ MS+ 20µM IBA	90.0	11.2±0.4	8.2	84.6	12.05±0.9	10.6

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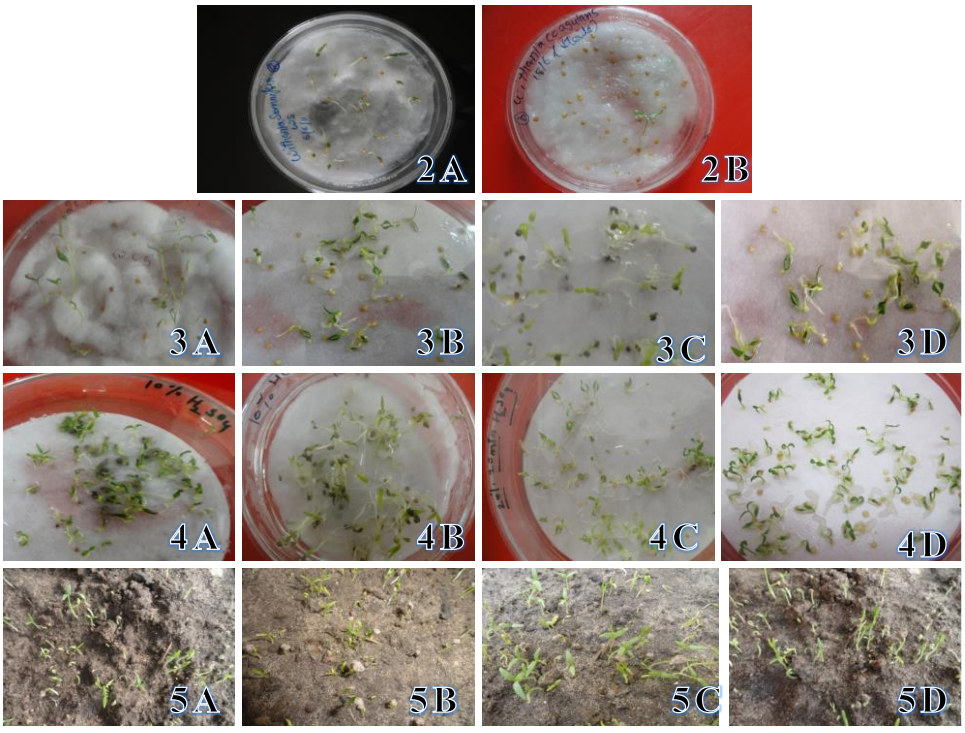

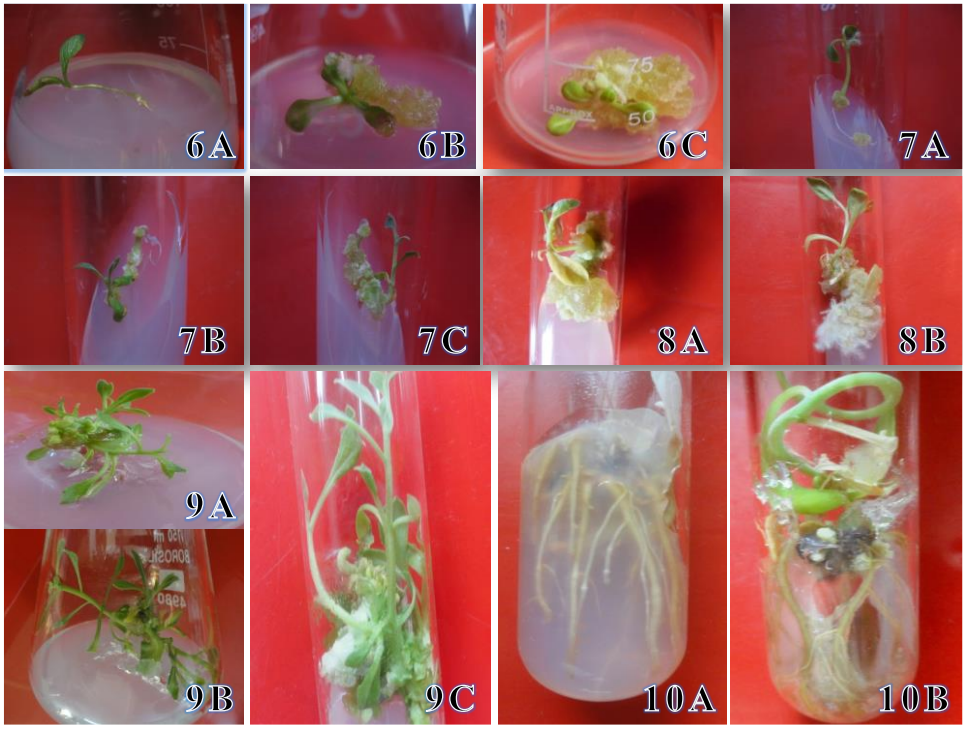

Fig.2A, 2B Germination of untreated seeds of *WS* and *WC*, **Fig.3A, 3B** Seeds of *WS* & *WC* (respectively) treated with 10% HCl, **Fig.3C, 3D** Seeds of *WS* & *WC* (respectively) treated with 10% H_2SO_4, **Fig.4A,4B** Seeds of *WS* & *WC*(respectively) treated with 20% HCl, **Fig.4C, 4D** Seeds of *WS* & *WC* (respectively) treated with 20% H_2SO_4, **Fig.5A, 5B** 10% HCl & 10% H_2SO_4 treated WS seeds germinated on soil , **Fig.5C, 5D** 10% HCl & 10% H_2SO_4 treated WC seeds germinated on soil, **Fig. 6(A-C)** Culture of seedlings of WS,(MS+NAA), **Fig.7(A-C)** Culture of seedlings of WS onto MS+BAP, **Fig. 8 (A-B)** Culture of seedlings of WC, (MS+NAA), **Fig.9 (A-B)** Regeneration from callus of WS, **Fig. 9C** Regeneration from callus of WC, **Fig 10(A-B)** *In vitro* rooting of WS & WC (respectively) onto ½ MS+IBA.