Studies on *in vitro* callus induction and proliferation from leaf explants of *Withania somnifera* (L.) Dunal

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Withaniasomnifera (L.) Dunal (Solanaceae) also known as Ashwagandha, is a valued medicinal plant known since antiquity. This plant is of high commercial value due to its medicinal property. This study shows the proper sterilizing protocol and effect of different combination and concentrations of plant growth regulators on the rate of callus induction and proliferation. Three types of combinations used were 1mg/l 2,4-D, 2mg/l 2,4-D and 0.2mg/l Kinetin and 0.5mg/l BAP and 0.5mg/l NAA. The callus growths were observed for around 6 weeks. It could be inferred that the combination of best suited plant growth regulators for callus induction and proliferation was 2,4-D and Kinetin.

Keywords: *Withaniasomnifera (L.) Dunal, explant, MS media, 2,4-D, IAA, BAP, Kin, callus induction, contamination.*

Abbreviation: *Murashige and Skoog media (MS), 2,4 Dichlorophenoxyacetic acid (2,4-D), Indole Acetic Acid (IAA), 6- Benzylaminopurine (BAP), Kinetin (Kin).*

I. Introduction

Withania somnifera (L.) Dunal has long been considered as an excellent rejuvenator, a general health tonic and a cure for several health complaints (Umadevi *et al.*, 2012). The drug has been positively tested for neuroprotective (Jain *et al.*, 2001; Ahmad *et al.*, 2005) properties. It is a commended genus described in Indian Ayurvedic System of medicine and also enlisted as important herb in Unani and Chinese traditional medicinal systems (Mir *et al.*, 2012).*W. somnifera* is widely distributed around the world and is mainly adapted to xeric and drier regions of tropical and subtropical domains, ranging from Canary Islands, the Mediterranean region and North Africa to South-west Asia (Mirjalili *et al.* 2009).

W. somnifera has not yet been assessed for the IUCN Red List. However, in Pakistan, *W. somnifera* is falling under criteria A of endangered category (Alam *et al.* 2009 and Haq 2011). Plant tissue culture offers opportunity for improvement of Ashwagandha in many ways such as, development of a mass propagation system, to obtain somaclones or mutants with desired chemical constituents, genetic transformations and *invitro* production of biologically active metabolites (Sharada *et al.*, 2008). *Withania* propagates vegetative in its natural state, but this propagation rate is much slower to meet demand of high-quality planting material for commercial cultivation. Its seeds are hard to germinate due to the presence of germination inhibitors (Karnik, 1978).Biotechnological techniques can help us to conserve this miracle plant. A swift and extremely effective method is micropropagation in which selected elite *Withania* plants, can be used as explants. This fast and competent regeneration protocol could be used for large production of selected cultivated varieties at commercial scale.

Many earlier studies have reported *invitro* propagation of Ashwagandha by using different explants, such as shoot tips (Rani *et al.*, 2014; Baba *et al.*, 2013; Sivanesan, 2007; Ray & Jha, 2001and Roja *et al.*, 1991), axillary bud (Rani and Grover, 1999), hypocotyl (Kulkarni *et al.* 2000), cotyledon (Kumar *et al.*, 2013), leaf (Joshi and Padhya, 2010 and Kulkarni *et al.*, 1996), seed (Supe *et al.*, 2006), cotyledonary leaf segments (Rani *et al.*, 2003), callus from leaf (Arumugam and Gopinath, 2013), shoot tip and root (Shrivastava and Dubey, 2007) and the nodal areas, (Kumar *et al.*, 2011). This study envisages an efficient method of callus induction from *in vitro* culture of leaf explants of *Withania somnifera*.

Materials and Methods

Saplings of *Withania somnifera* was collected from Roy Nurserylocated at Tribeni, Hoogly, West Bengal, Gour-Nitai Nursery, Dum Dum, Kolkata, West Bengal and a nursery in Sealdah, Kolkata, West Bengal. Plants maintained in earthen pots at normal environmental conditions in polyhouse. Plants were regularly watered and treated with fertilizers.Insecticides like 0.01% v/v Rugor and fungicides like 0.001% w/v Bavistin were used in regular interval to keep the plant uninfected and disease free. Suitable juvenile young leafexplants, that is, the 3rd leaf from the apex of the plant were selected from these saplings after a month.

Glass goods and instruments were sterilized by autoclaving in 15lb/inch^2 pressure at 121^{o}C for 15 mins and stored in incubator for later usage.The culture room and laminar airflow were exposed to UV light for 30 mins. After UV treatment, the hood of the laminar airflow was kept open to allow the air to stabilize. The table of the laminar air flow was cleaned with alcohol. Scalpels and forceps were dipped in 70% v/vrectified spirit (Bengal Chemical Ltd.) poured in coupling jar prior to work.

Young leaves were collected and washed thoroughly under running tap water for 15 minutes. Washed explants were treated with 1% v/v ionic surfactant (Tween 20) for 5 minutes. Then the explants were washed again under tap water until there was no trace of detergent. The explants were then washed again in 0.1% w/v $HgCl_2$ for 2 minutes. Again, the explants were washed in double distilled water thoroughly to remove traces of $HgCl_2$. The explants were then cut into different sizes on an autoclaved petridish and excess water was blotted off using blotting paper.

The cut explants were then transferred to culture tubes containing MS media (Murashige & Skoog, 1962) with three combinations of growth regulators: 2,4-D, 2,4-D + Kin and BAP+NAA.

Results and Observation

Leaf explants of approximately 0.7- 0.9 cm^2 were taken, the callusing did not initiate for a long time, ultimately the most of the leaf explants degenerated. When leaf explant of 0.2-0.3 cm^2 were taken, all the explants were unresponsive and quickly degraded. But, intermediate 0.5 cm^2 size of the explants, were taken, appeared to be comparatively more responsive. Further, when older leaves from 5-6th leaf positions from apex were taken up, callusing were not observed. While leaves from 1st position from apex when taken,

it was difficult to work, moreover, being smaller in size meristematic tissues could not be dissected out properly. Hence, the optimum responsive explant was obtained from 3rd leaf of 0.5 cm^2 of size.

Three different durations of $HgCl_2$ treatment were applied for surface sterilization, mainly 2 mins, 3 mins and 10 mins. It was observed that the highest contamination percentage was obtained in 2 mins incubation, i.e., 44.44%, However, 10 mins of incubation of surface sterilization showed 19.05% contamination but most of the explants were not responsive in culture. However, the duration of 5 mins, which showed 28.60% contamination, but rest of the uncontaminated explants responded better in culture. [Fig. 1(a)]

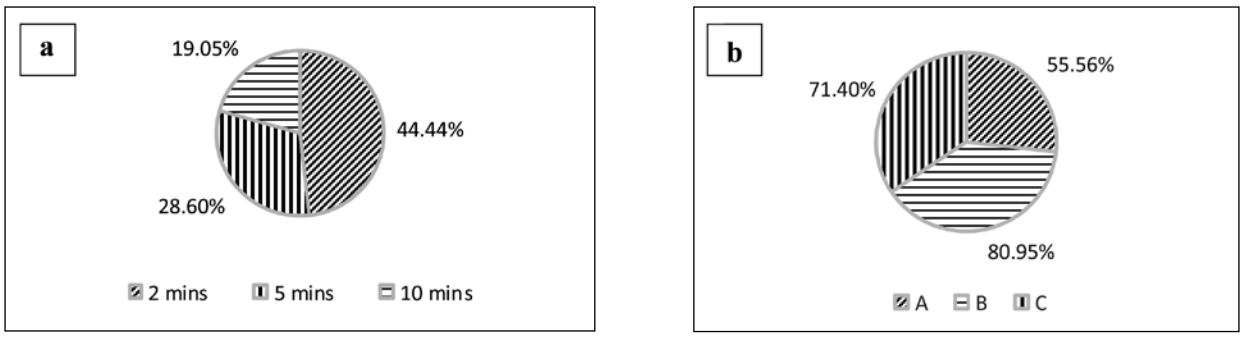

Fig 1. Pie Charts showing **(a)** Percentage of contamination, **(b)** Percentage of callus induction in various combinations of growth regulator supplemented MS media after 42 days **A**: 2,4-D(1mg/l), **B**: 2,4-D(2mg/l) + Kin(0.2mg/l),**C**: BAP(0.5mg/l) + NAA(0.5mg/l)

The explants initially swelled and appeared contoured after 7-10 days depending upon their growth regulator combination [Fig. 2(A, I)]. Thereafter, small, round colonies of micro callus started appearing withing 25 days [Fig. 2(B, F, J)], which further proliferated covering the entire cut surface of the explant [Fig. 2(C, G, K)]. After 42 days in culture, in each combination different quantum of callus was generated [Fig. 2(D, H, L)]. This pattern of callus induction was also reported by Joshi & Padhya, (2010) and Srivastava, (2007).

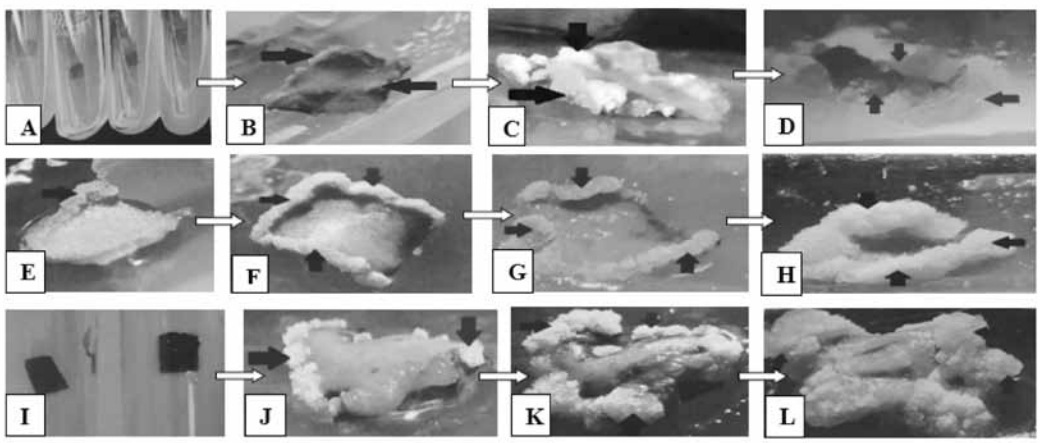

Fig 2. Pictures of gradual callus induction of leaf explants of *W. somnifera* in MS media supplemented with various growth regulators, taken through Olympus Stereo-microscope in 4X magnification except **A** and **I**. In MS + 2,4-D(1mg/l), **(A)** culture was initiated, **(B)** first callus initiation was observed around 25th day, **(C)** callus started to proliferate**, (D)** development of a dry yellowish callus.In MS + 2,4-D(2mg/l) + Kin(0.2mg/l), **(E)**callus initiation started by 14th day, kept on proliferating, **(F)** proliferation of callus on the edges of explant, **(G)** further swelling and proliferation of callus**, (H)** development of a friable and white callus. In MS + BAP(0.5mg/l) + NAA(0.5mg/l), **(I)** culture initiation, **(J)** callus initiationobserved on 18th day, **(K)** further swelling and callus proliferation, **(L)**, development of a compact, yellowish callus.

It was observed that when callus induction in MS medium supplemented with various combination of growth regulators were conducted, the responsive explants in general transformed to colourless tissues which subsequently became whitish swelled outgrowths. After culturing for 42 days, amongst other combinations, 80.95% of callus induction, followed by combination of BAP(0.5mg/l) + NAA(0.5mg/l), which gave 71.4% and 2,4-D(1mg/l) gave 55.56% [Fig. 1(b)]. Even the early callus induction efficacy was higher (14 days) in growth regulator combination of 2,4-D (2mg/l) + Kin (0.2mg/l), followed by 18 days for BAP (0.5mg/l) + NAA (0.5mg/l) and 25 day for 2,4-D (1mg/l) [Fig. 3(a)]. Therefore, combination of 2,4-D and Kinetin proved to be best for early callus induction and high percentage of response.

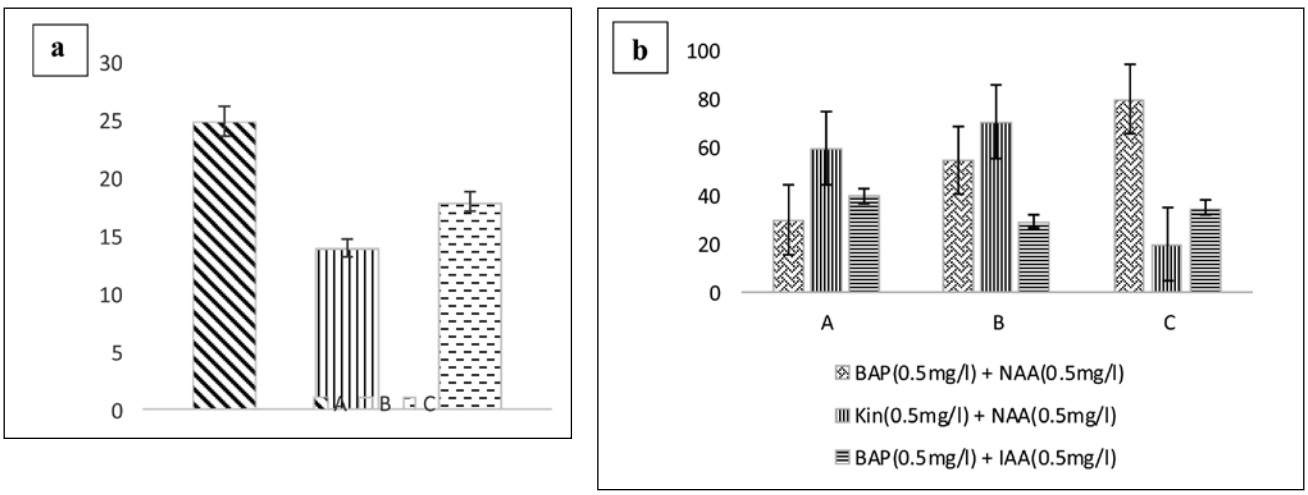

Fig 3: (a) Graphical representation of average number of days taken for callus initiation within a total observation period of 42 days, **(b)** Graphical representation of callus proliferation over a totalobservation period of 42 days in MS media supplemented with various growth regulators.

A: 2,4-D(1mg/l), **B**: 2,4-D(2mg/l) + Kin (0.2mg/l) and **C**: BAP(0.5mg/l) + NAA(0.5mg/l)

After 6th week, three types of callus, namely dry callus, friable callus and solid callus were observed. Callus obtained from MS media supplemented with 2,4-D was observed to be dry in nature. While callus obtained from combination of 2,4-D + Kin appeared friable and BAP + NAA was compact and hard.Friable callus generally is suitable for further subculturing, organogenesis and cell culture initiation. Therefore, the combination of 2,4-D + Kin was best for callus formation as it resulted in friable callus.

The proliferated callus was further sub-cultured in different sets of growth regulator combination to observe the rate of callus proliferation. Callus proliferated in the media supplemented with 2,4-D, 2,4-D + Kin and BAP + NAA were further sub-cultured in 3 sets of growth regulator combinations, namely BAP(0.5mg/l) + NAA(0.5mg/l), Kin(0.5mg/l) + NAA(0.5mg/l) and BAP(0.5mg/l) + IAA(0.5mg/l)[Fig. 3(b)]. In 2,4-D cultured callus, BAP+NAA showed minimum callusing of 30%, Kin+NAA showed moderate callusing (60%) and BAP+IAA showed minimum (40%). However, 2,4-D+Kin, BAP+NAA showed moderate callusing, 54.76% while Kin+NAA showed 70.59% callusing and BAP+IAA showed minimum callusing, 29.41%. In case of BAP+NAA, the first combination of BAP+NAA showed 80% callusing, Kin+NAA showed 20% and BAP+IAA showed 35.08%.

Discussion

The explants when derived from third leaves of juvenile plants, showed good response after surface sterilization for 5 mins in $HgCl_2$. The study also found that 2,4-D + Kin combination gave maximum response for dedifferentiation and subsequent callus induction. Manickram *et al*., (2000) reported that 2,4-D when used singly turned out to be potent growth regulator for callus induction from leaf explant of *Withania* too. Similar results were observed with Kin using leaf explant for shoot bud induction and *in vitro* proliferation by Sivanesan & Murugesan, 2005. However, after callus initiation, the further development was found to be optimum when BAP + NAA combination was used with MS medium. Detailed study by Adhikari and Pant, (2013) showed that BAP (0.5mg/l) + NAA (1.5mg/l) was best among other concentrations of same combinations of growth regulators supplemented in MS medium. Ghimire *et al*., (2010) emphasized on the use of NAA for callus maintenance for future regeneration and shoot bud induction from petiole and leaf explants. Therefore, after initial induction of callus, the growth regulators are to be substituted for maintenance and proliferation during subsequent sub-culture as each growth regulators perhaps have specific physiological role in tissue orientation, redifferentiation and growth.

Acknowledgement

We would like to express our sincere gratitude to the Dr. Arpita Mukherji, Principal, Scottish Church College, for supporting and providing us the facility to work on the dissertation study for 2 years.

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