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OPTIMIZATION OF *IN-VITRO* PROPAGATION PROTOCOL FOR *PLANTAGO OVATA* Forsk

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The present investigation is an effort to establish callus formation and plant regeneration from cultured upper and lower hypocotyl explants of different varieties of *Plantago ovata.* Adventitious shoots were regenerated from cultured upper as well as lower hypocotyl explants excised from two to four weeks old *in vitro* grown seedlings of *Plantago ovata* on MS medium containing various concentration and combination of IAA, IBA, NAA, 2,4-D, BAP and KN. Multiple shoot regeneration was observed from upper hypocotyl explants. Complete plant regeneration was observed from upper hypocotyl in MS media supplemented with low concentration (0.5 mg/L) of IBA, IAA and NAA separately and in combination of BAP (2.0 mg/L) and NAA (0.5 mg/L). When IAA (0.5, 1.5, 2.5, 5.0 mg/L), IBA (0.5, 1.5, 2.5, 5.0 mg/L) and NAA (0.5, 1.5, 2.5, 5.0 mg/L) were used separately in the media, there was remarkable growth of roots from both upper and lower hypocotyl parts. In lower concentration of IBA (0.5 mg/L) white and nodular callus growth with root formation was observed. 2,4-D induced callusing in both the explants at uniform rate with the concentration ranging from 1.5 mg/L to 2.5 mg/L.

Keywords : *Plantago ovata,* medicinal plant, micro-propagation, callus formation, IBA, IAA, NAA,2,4-D, BAP.

Plantago ovata Forsk, commonly known as Isabgol, is an annual herb belongs to the Plantaginaceae family. Isabgol has been used in medicine since ancient time, but it has been cultivated as a medicinal plant only in recent decades (Rout *et al.* 2000, Handa and Kaul 1999, Lal *et al.*1999, Rahn 1996). It is a large genus of herbs, distributed mostly in the temperate regions and a few in the tropics. The genus *Plantago* of family Plataginaceae include some 200 species. Among these species 10-14 are natives of India. It's seeds and husk are the highest export earning medicinal plant product. India continue to hold a monopoly in its production and trade in the world (Haines 1921-1925).

Gujrat Isabgol 1 and 2, Haryana Isabgol 5 and 34, Niharika are different varieties of *Plantago ovata* which are grown extensively in Haryana, Gujrat and Rajasthan. Seeds are used to prepare Psyllium powder which is the strongest natural dietary fiber and bulk in the diet which helps to reduce the intake of starchy foods, enhances gastrointestinal function and prevents constipation. Due to these properties it is largely used in pharmaceutical industries and in preparation of different system of medicine. At present this plant is not grown commercially in the state of Jharkhand. In India the crops are grown in the winter season and cool and dry weather with sandy and silty soil are favourable for its growth and germination begins in four days after sowing of seeds. The seeds of isabgol contains mucilage, fatty oil, large quantities of albuminous (C_{13} $H_{19}O_8H_2O$) and a pentose sugar (Jamal *et al.*1987, Chavallier 1996, Karimzadeh and Omidbaigi 2004, Zargari 1990, Ansari and Ali 1996, Chopra *et al.* 1986). The seed husk is diuretic, alleviate kidneys and bladder complaints, gonorrhea, arthritis and hemorrhoids (Ansari and Ali 1996, Chopra *et al.* 1986). Psyllium seed husk is 34% insoluble fiber and 66% soluble fibre that make it a valuable food additive. The seed husk is not only a highly effective laxative but it is also used in lowering blood cholesterol levels (Dhar *et al.* 2005, Chopra *et al.*1986).

Plantago ovata propagates in nature by seeds and not by other parts such as root, stem and leaf. Therefore this plant has been selected and subjected to experimental morphogenic studies which is less studied for this plant.

MATERIALS AND METHODS

Culture Medium

MS (Murashige and Skoog, 1962) medium was used as basal nutrient medium in present investigation with 3.0% sucrose as carbon

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source. The chemicals used for preparing various media were of analytical grade from Loba, Merck and Sigma. Medium was homogenized by boiling and by continuous stirring and pH of the medium was adjusted to 5.8 before adding 0.8% agar. Desired concentration of growth regulator was added and mixed properly. 15-20 c.c. medium was poured into culture tubes (1.5 x 2.5 cm) which were washed thoroughly and rinsed in distilled water and oven dried. Sterilization of the medium was done at 121ºC for 15-20 min under 1.4 kg/cm2. Culture Material and explant source

For the present study mature seeds of *Plantago ovata* were collected from Anand Agricultural University, Gujrat during the month of Oct-Jan. Seeds of other varieties were obtained from Hissar Agricultural College, Haryana.

Seeds were washed thoroughly in tap water and treated with cetavelon (1:100) for 5 minutes. This was followed by washing with running tap water and then by distilled water. Surface sterilization was carried under aseptic condition in laminar air flow cabinet and treated with 0.2% $HgCl_2$ for 1-2 minutes and rinsed thrice thoroughly with sterile distilled water. Now these were cut into small segments of 0.5-1.0 cm length and made ready for inoculation.

Seedlings were grown in culture tubes containing MS medium without plant hormones. For the subsequent treatments, upper and lower hypocotyl explants from approximately 2-4 week-old-seedlings which were grown from seeds were cut into 0.5 or 1 cm and then cultured on MS medium containing IBA(0.5 – 5.0 mg/L), IAA(0.5 – 5.0 mg/L), NAA(0.5 – 5.0 mg/L), BAP(2.0 mg/L)+NAA(0.5 mg/L), IBA (1.5 mg/L) + 2,4-D (0.5 mg/L)for direct regeneration and callus induction.

Culture Condition

Floor surface transfer area was sterilized with UV light and by swabbing with 95% ethyle alcohol. Autoclaved inoculation tools were used for transferring explants onto the medium. Culture tubes were maintained in culture cabinet in diffuse fluorescent light at temperature 25±2ºC and at 50-60% humidity with 16 hour photoperiod. Each experiment with a minimum of 10 cultures was repeated 2-3 times and were observed every day for their morphological response.

For the acclimatization and transfer of plantlets to soil, the *in-vitro* grown rooted plantlets were transferred to plastic cups containing sterilized sand and coco-peat(1:1) and were nourished time to time with ½ strength liquid MS medium. The cups were covered with plastic cover and kept in the culture room maintained at 25±2º C under cool fluorescent light with 16 hour photoperiod. After 5 weeks rooted plantlets were transferred to garden soil for acclimatization.

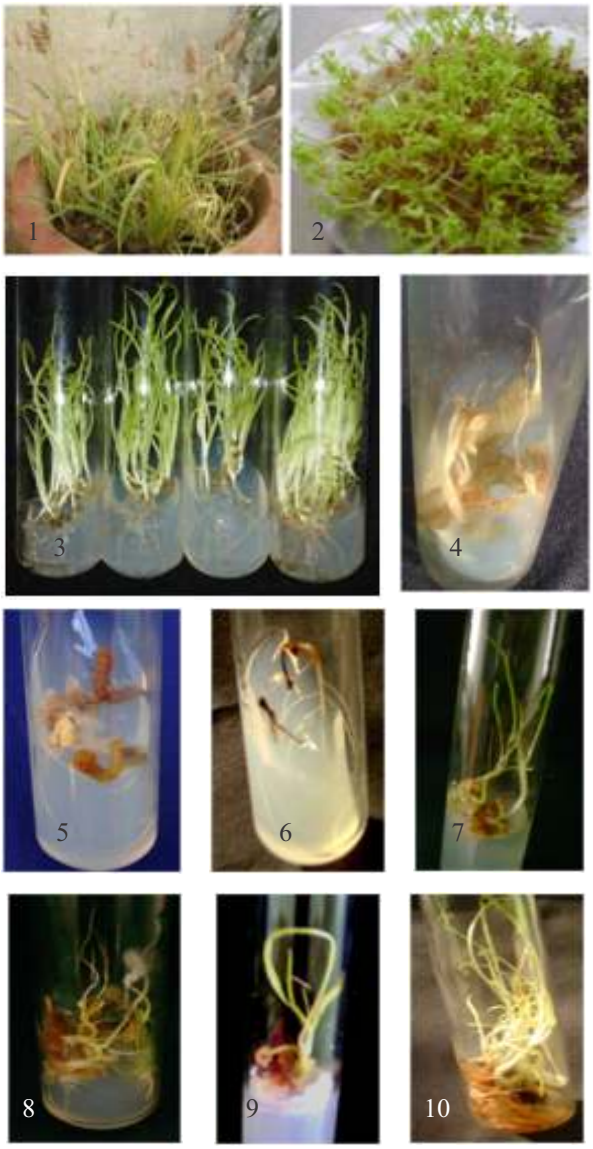
Data analysis

During present investigation data presented in the tables are the treatments consisting of 10 replicates(culture tube) and the experimental unit was a single explant per tube. Data were analyzed using standard error method.

RESULTS

During present investigation, among all the varieties tested for their germinability, GI-2 variety showed best germination in field as well as culture condition. Therefore GI-2 variety was selected as the experimental material. During present work when mature fresh seeds of GI-2 variety were implanted on plain MS medium, 80.71± 1.66% germination was observed. After 4-5 days of implantation on plain MS medium epigeal germination was observed with well developed tap root system. Various plant parts of these *in vitro* grown seedlings were cultured on MS medium supplemented with different growth regulators.

Seeds also implanted on MS medium supplemented with different concentrations (0.5 to 5.0 mg/L) of auxin showed different



Figures 1-10 *Plantago ovata*. **1.** Mature plants *Plantago ovata* Forsk. GI-2 variety growing in my garden pot showing spike inflorescence. **2.** Ten days old seedlings of GI-2 variety grown in petriplate. **3.** Three weeks old seed culture on plain MS showing germination. **4.** Four weeks old upper hypocotyl culture on MS + 2,4-D (2.5 mg/L) showing callus. **5.** Five weeks old lower hypocotyl culture on MS + NAA(2.5 mg/L) showing brown and white callus with roots. **6.** Seven weeks old culture of lower hypocotyl on MS +IAA (1.5 mg/L) showing well developed tap root. **7.** Six weeks old upper hypocotyl culture on MS + IBA (0.5 mg/L) showing plantlets with little callus formation. **8.** Seven weeks old culture of upper hypocotyl on MS +IBA (1.5 mg/L) + 2,4-D (0.5 mg/L) showing simultaneous development of shoot, root and callus. **9.** Six weeks old culture of upper hypocotyl on MS +BAP (2.0 mg/L) + NAA (0.5 mg/L)showing plant regeneration and callusing from the tip of the tillers. **10.** Seven weeks old culture of upper hypocotyl on MS + IAA (0.5 mg/L) showing profused growth of root and shoot with callusing from regenerated roots.

Figure 11 Column graph showing number of tillers from *in-vitro* raised upper and lower hypocotyl of *Plantago ovata*.

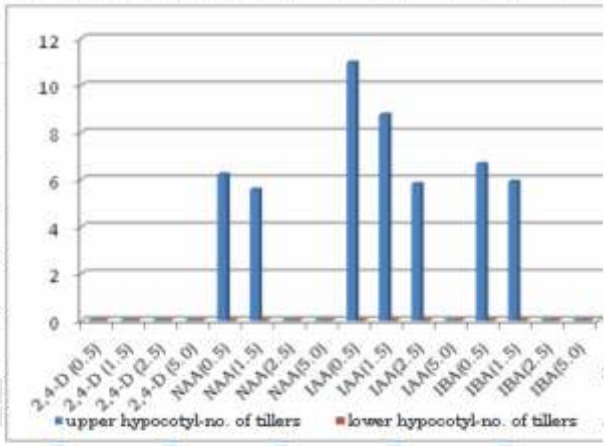


Figure 12 Column graph showing length of tillers from *in-vitro* raised upper and lower hypocotyl of *Plantago ovata*.

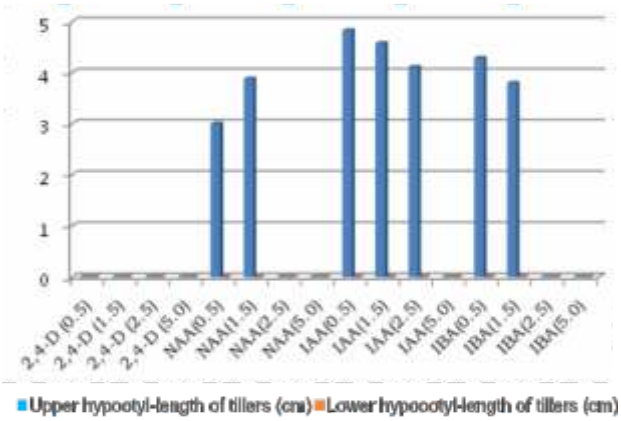


Figure 13 Column graph showing no. of roots from *in-vitro* raised upper and lower hypocotyl of *Plantago ovata*.

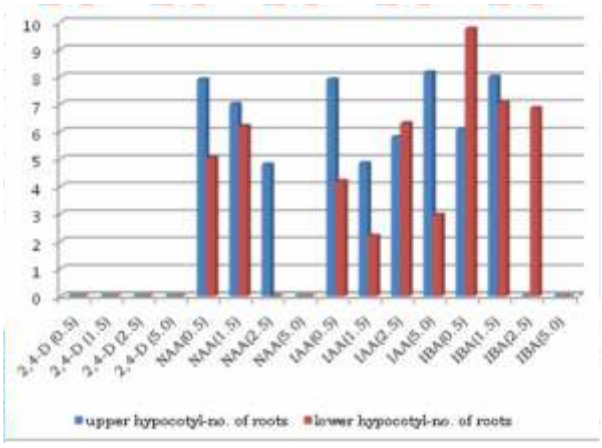
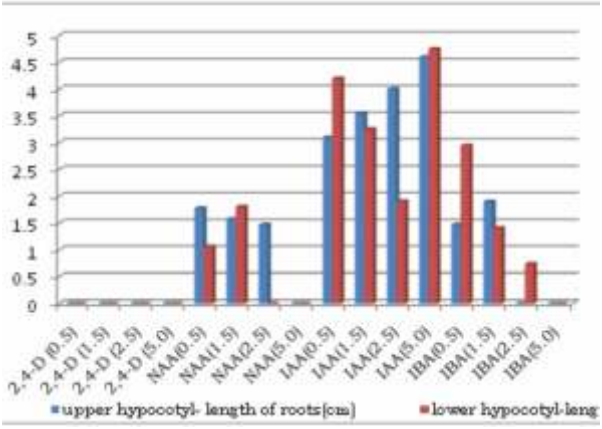


Figure 14 Column graph showing length of roots from *in vitro* raised upper and lower hypocotyl of *Plantago ovata.*



types of response. In lower concentration of 2,4-D (0.5 mg/L) one to two tiller formation with hypertrophy was observed. In higher concentration (1.5 to 2.5 mg/L) different types of calli were observed. There were no root and shoot regeneration was observed when seeds were inoculated with MS and 2,4-D. Effect of various auxins supplemented with MS media on direct regeneration of plantlets from various parts mainly upper and lower hypocotyl segments of *in vitro* obtained seedlings of *Plantago ovata,* has been recorded (Fig. 11,12,13 and 14).

In low concentration of IBA (0.5 mg/L) white and nodulated callus with root formation was observed from lower hypocotyl while in different concentration of IBA (0.5, 1.5, 2.5 mg/L) shoot along with root and callus was observed from upper hypocotyl(Fig.7) During present investigation only callus formation was observed in higher concentration of IBA (5.0 mg/L) from both the explants showing direct plantlet formation with average number of tillers 6.65 ±0.22 per explants and average length of tillers 4.29±0.16. During present investigation when IAA (0.5 mg/L) used separately was the most suitable growth regulator for direct plant regeneration from upper hypocotyl explants of in vitro grown seedlings (Fig.10) but in higher concentration of IAA (5.0 mg/L) profused growth of callus with number of root formation was observed from lower hypocotyl explants. When 14 days old *in vitro* grown upper hypocotyl was cultured on medium supplemented with IBA (1.5 mg/L) + 2,4-D (0.5) simultaneous development of shoot, root and callus was noticed (Fig. 8). The upper hypocotyl and lower hypocotyl segments cultured on MS medium supplemented with different concentration of NAA (0.5 to 5.0 mg/L) exhibited callus formation as well as shoot and root formation from entire surface. The effect of combination of auxin and cytokinin on MS medium was very significant. When upper hypocotyl segment was cultured on MS media supplemented with BAP (2.0 mg/L) and NAA (0.5 mg/L), direct plant regeneration with little callus from the tip of the tillers was obtained (Fig.9)

DISCUSSION

The expressions of totipotentiality also vary with the physiological state of the explants derived from the same plant (Gamborg *et al.* 1974a). Meristematic region responded well to yield calli having higher regenerability. In the present investigation upper hypocotyl with shoot apices was the best explant for direct plant regeneration in *Plantago ovata.* Whereas callusing was induced from seeds, upper and lower hypocotyl part of *Plantago ovata*. Lower hypocotyl explants measuring 8-10 mm of 8-20 days old seedlings were the best suited explants for callus production. It was observed that seeds and *in-vitro* formed lower hypocotyl which were neither very young nor very old, were the most suitable portions of the seedling for callus induction in *Plantago ovata.*

Complete plant regeneration with two to three branches from upper hypocotyl explants (20 days old) were observed on MS media supplemented with IAA (0.5 mg/L) (Fig.7) during present investigation and these results are not similar to the findings of previously reported by Barna and Wakhlu (1988), Wakhlu and Barna (1989), Pramanik *et al.* (1995).

In the present investigation calli were initiated

on MS media supplemented with various concentration of 2,4-D, IAA, NAA and IBA in *P. ovata.* Nodular and white callus in cultures of lower hypocotyl of *Plantago ovata* was observed on medium supplemented with IBA (0.5 mg/L).

Callus initiation was observed from the cut portions of the lower hypocotyl part on the medium with combination of IBA (1 mg/L) and KN (1.5 mg/L). 6-7 days old seedlings gave rise the shoots at the highest frequency of 96-100% and also produced highest number of shoots per explants with 4-5 shoot from hypocotyl explants (Chattopadhyay *et al.* 1995). Irrespective of the type of explants used, its age plays a critical role in determining the response *in vitro.* Very young explants often fail to show any growth whereas older ones produce either root forming or non regenerable callus.

Concentration of different plant growth regulators in the media significantly influenced shoot regeneration from upper and lower hypocotyl explants. The greatest multiplication rate obtained in present investigation was found in upper hypocotyl part of *P. ovata* cultured on MS media containing IAA (0.5 mg/L), IBA (0.5 mg/L), NAA (0.5 mg/L) where shoot regeneration was better than lower hypocotyl. It shows that upper hypocotyl had better regeneration potential in *P. ovata.* Previously direct organogenesis was occurred in case of *P. lanceolata* from segments of leaves and roots on MS containing (2.0 mg/L) and KN (2.0 mg/L) (Budzianowska *et al.* 2004). Similarly, shoot regeneration from hypocotyl and cotyledon explants of *P. afra* under various concentrations of BAP + IBA was previously recorded (with best multiplication effect by using seedling tips of *Plantago ovata* with MS medium enriched with growth regulators IAA (0.2 mg/L) and BAP (0.5 mg/L) (Pramanik *et al.*1995). During our present work with *P. ovata* greatest effect of regeneration was achieved from hypocotyls. Presence of 2,4-D with KN was essential growth regulator for callus initiation which was followed by addition of BAP and NAA in subculture medium for producing embryogenic callus (Sarihan *et al.* 2005). But in present work with *Plantago ovata* direct plant regeneration was obtained when *in vitro* grown upper hypocotyl part was cultured on BAP (2.0 mg/L) + NAA (0.5 mg/L). In *Plantago maritima* it was reported that BAP promoted shoot formation in a concentration 0.1, 0.2, 1.0 and 2.0 mg/L (Chang and Loci 1996). However, this combination has frequently been found favourable for proliferation of shoots of the same and other species (Makowezynska and Andrzejewska 2000, Mederos 1994, Mederos and Schobert 1995).

From lower hypocotyl of *Plantago ovata* white, nodulated callus was achieved whereas upper hypocotyl was suitable for multiple shoot regeneration with roots. However in present work when these explants were cultured on MS medium supplemented with different growth regulators produced shoot, root and little callus. The callus showed a differential response according to the growth regulators used. The present study has thus established a method for regenerating plants from upper hypocotyl part of *P. ovata* and opens up the possibility of plantlet formation without using seeds.

In general, high concentration of cytokinin and low concentration of auxin are required in a medium to promote the induction of shoot morhogenesis (Kohlenbach 1997) and this was found true in the present study for shoot regeneratiom using *in vitro* explants of *Plantago ovata.* Auxins (NAA, IAA and IBA) in high concentration induced profused callus rather than roots from the base of the shoots (Mallikarjua and Rajendrudu 2007).

In present study IAA was found more effective than that of IBA and NAA while 2,4-D was not effective for root formation from the upper and lower hypcotyl explants of *P.ovata. In vitro* root formation of *P. ovata* was significant part of the present work.

Profused root growth in culture is probably due to the high content of endogenous plant growth regulators. It was concluded that the presence of BAP with NAA promoted precocious root development in *P. ovata,* which could be suppressed by replacing NAA with IBA. These results are also recorded in *P. afra* (Sarihan *et al.* 2005) and *P. lanceolata* (Khawar *et al.*2005).

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