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Effect of hygromycin on peanut germination and its application in *Agrobacterium*-mediated genetic transformation

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Abstract
To overcome the laborious screening tasks in *in planta* transformation, *Agrobacterium* infected seed parts mainly embryonated cotyledons and embryos can be passed through the selection of antibiotics for a very short duration for successful identification of transformants. Hygromycin B more toxic than the existing antibiotics and has been used as a selective agent in many crop plants. With the increase in hygromycin concentrations, a decline in the survival frequency of explants was observed. The threshold concentration of hygromycin for the selection of transformants was optimized using embryonated cotyledon and embryo on MS media containing different concentrations of hygromycin. It was observed that 20 mg/l hygromycin was threshold concentration for the selection of embryonated cotyledons and 15 mg/l for embryos. These concentrations suppress the growth of peanut plants. Therefore, hygromycin at 20 mg/l for embryonated cotyledons and embryos at 15 mg/l could screen hygromycin-tolerant transgenic peanuts.

Keywords: Peanut, hygromycin, in planta transformation, embryonated cotyledons and embryos

Introduction
Peanut (*Arachis hypogaea* L.) is an important principal commercial oilseed crop rich in high quality edible oil (43-55%), digestible protein (25-30%) and carbohydrate (10-20%) [1]. It plays an important role in ameliorating livelihoods, nutrition and economy of many countries. Peanut is a good source of vitamins and inorganic ions important for human nutrition and can help in preventing malnutrition [2]. With the rise in the demand across the world, there exists a pressure on the production of peanut to meet the requirement. Conventional approaches to the genetic improvement of peanut were hampered by low genetic diversity and polyploidy [3, 30]. Therefore, the use of biotechnology procedures can assist in the genetic improvement of peanut. Gene discovery is followed by the investigation of specific functions of the individual genes and their characterization [4]. For generating transgenic plants, a plant vector harboring a selectable marker gene plays a vital role in efficient selection of transformants. Therefore, efficient and reproducible selection of transformed tissues and cells plays a crucial role in a successful transformation system. Though there are different selective markers, usually antibiotics are used to select transformants and nowadays hygromycin resistance gene appears to be the best selection marker as hygromycin is more toxic than the existing antibiotics. Hygromycin B is one of the aminoglycosidic antibiotics to kill bacteria, fungi, and higher eukaryotic cells by inhibiting protein synthesis by mistranslation and interferes with protein translocation [5]. Hygromycin resistance gene encodes the enzyme hygromycin B phosphotransferase that phosphorylates the antibiotic hygromycin and confers antibiotic resistance to transformed cells harbouring the hygromycin resistance genes in eukaryotes and prokaryotes [6, 7, 8]. Hygromycin B has been employed as a selective agent in both dicots and monocots. The optimal concentration for selection varies with plant species and needs to be determined empirically.

Many legume transformation studies have established *Agrobacterium tumefaciens* mediated development of transgenic soybeans [9, 10], chickpeas [11] and pea [12, 13, 14, 15]. Most strategies using *Agrobacterium*-mediated gene transfer have been performed under *in vitro* culture conditions and require identification of competent tissues for transformation and development of tissue culture systems that convert these tissues into plants [16, 17, 18, 19, 20, 21, 22, 23]. To meet the raising market demand, the genetic improvement of peanut by recombinant technology is necessary. Minimizing the role of tissue culture in transformation procedure will be favorable. Transgenic development has been successful using embryonic axes, apical meristem and seedling explants [24, 25, 26, 27, 28]. The limitation of current peanut transformation is the requirement for effective screening of transgenics produced by *in vitro* genotype-independent

regeneration protocols. Screening false positives along with transgenics in *in planta* transformation is a laborious task [27]. To overcome this task, *Agrobacterium*-infected seed parts, mainly embryonated cotyledons and embryos can be passed through the selection of antibiotics for a very short duration for successful identification of transgenic plants. The purpose of this study was to evaluate the effect of hygromycin concentration on commonly used seed parts like embryonated cotyledons and embryos for *in planta* transformation to develop an efficient selection protocol based on hygromycin-B. So far, no report has been published on the threshold concentrations of hygromycin on embryonated cotyledon and embryo of peanut to exploit their usefulness in *in planta* transformation. In this study, we investigated threshold levels and also the effects of hygromycin on the height of shoots, number of nodes per plantlet, length of the main root, lateral roots length and the number of lateral roots per seedling.

Materials and Methods

Plant materials: Peanut (*Arachis hypogaea* L. var. JL-24) seeds were obtained from ICRISAT, Hyderabad, India. The seeds were surface sterilized with 70% (v/v) ethanol for 1 minute, followed by 0.1% (w/v) mercuric chloride solution for 8 minutes, rinsed 5 to 7 times and soaked overnight in sterile distilled water. Embryonated cotyledons and embryos were carefully removed by- separating one cotyledon and both cotyledons respectively, at the site of their attachment to the primary axis.

Assay of the effects of different concentrations of hygromycin on peanut germination and seedling growth

The internal resistance of peanut embryonated cotyledons and embryos to hygromycin was tested by inoculating them in test tubes containing half-strength MS medium [half-strength MS salts [29], 100 mg/l myoinositol, 3% sucrose, 8.5 gm/l agar, pH 5.6 prior to the addition of agar] with different concentrations of hygromycin. Embryonated cotyledons were subjected to hygromycin concentrations of 1-10mg/l, 15mg/l, 20mg/l, 25 mg/l, 30mg/l, 35mg/l, 40mg/l and embryos on 1-10mg/l, 15mg/l, 20mg/l, 25mg/l, 30mg/l and 35mg/l respectively. Embryonated cotyledons and embryos inoculated in the medium without hygromycin served as controls. Following different time intervals of culture, the morphology of seedling growth on different hygromycin concentrations was studied. Meanwhile, height of shoots, number of nodes per plantlet, length of main root, lateral roots length and the number of lateral roots per seedling were analyzed during the period of 15 days.

Assay of effects of different concentrations of hygromycin on the root of *Arachis hypogaea* seedling

Seeds of *Arachis hypogaea* L. were sown on MS with 0-5 mg/l hygromycin and after 7 days seedlings were transferred onto MS medium without hygromycin. Seeds without hygromycin in the medium served as controls.

Culture conditions: All the cultures were maintained at 25±2 ^{o}C under 16/8-h light/dark cycle with a light intensity of 60 µmol photon m^{-2} s^{-1} supplied by cool-white fluorescent lamps.

Experimental design and statistical analysis

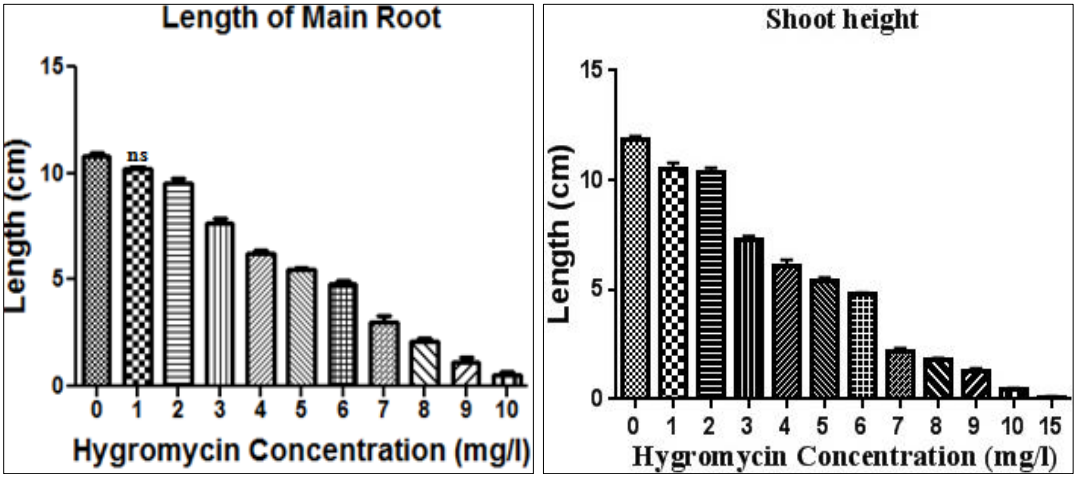
The experimental design was completely randomized design. Each experiment was repeated three times. Data represented average values from three replicates ± SE. Results were analyzed using one-way analysis of variance (ANNOVA) and statistical differences among treatment means were compared using Duncan's Multiple Range Test (5%) using Graph Pad Prism Software (v.6.05), Inc.2015.

Results and Discussion

Assay of the effects of different concentrations of hygromycin on peanut germination and seedling growth

When cultured for 5d, the changes in the embryonated cotyledons at different concentrations of hygromycin were as follows. (A) At 1-3 mg/l of hygromycin, the hypocotyl-radical was swollen, primary root elongated with small lateral roots and plumule turned to green; (B) at 4-10mg/l of hygromycin, roots were elongated, dwarfed shoot and plumule were green; (C) at 15 mg/l, roots initiated mainly and plumules turned green; (D) at 20-40mg/l no change in the explants and color was turned to brown.

After 15 days of culture of embryonated cotyledons on MS with different concentration of hygromycin, some began to turn brown and necrotic. At 15d of culture, the following results were obtained: (a) at 1-6 mg/l hygromycin, primary roots elongated with many lateral roots and shoots were well developed; (b) at 7-10 mg/l hygromycin main roots elongated without any lateral roots and shoot growth was observed (c) at 15 mg/l hygromycin, roots initiated and turned brown without any further growth and plumule turned slightly green (d) at 20-40 mg/l, no change in the size, but cotyledon, radical and plumule turned brown and necrotic. In controls, shoots were developed of three internodal lengths and main tap root was developed with profused lateral roots. Figs. 1 and 2 show that hygromycin was toxic to peanut seedlings, and hygromycin concentrations greater than 20mg/l inhibited the development of roots, hypocotyls, and plumule or shoot formation (Fig. 1 and 2).



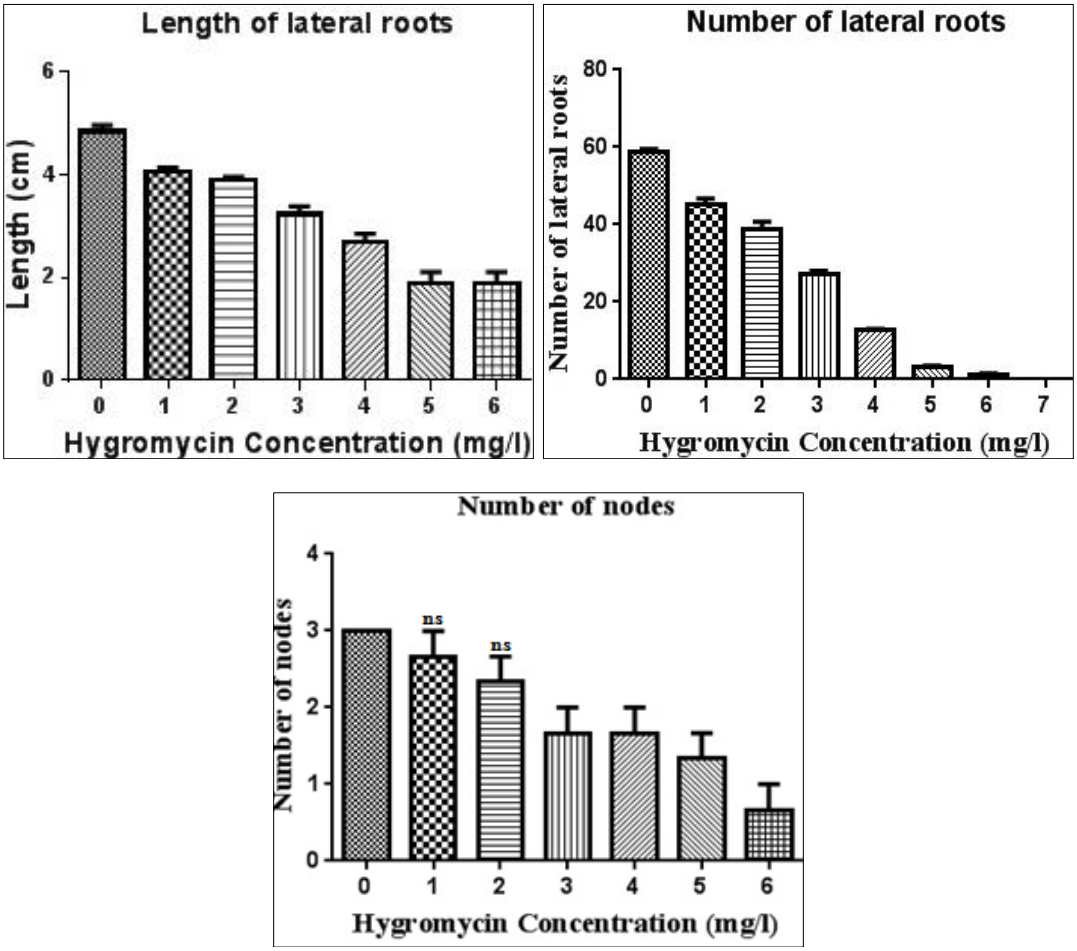


Fig 1: Dosage effect of hygromycin on growth of embryonated cotyledons. Data represented means ± SE of three replications. Bars represented with 'ns' are not significantly different at P <0.05 by Duncan's multiple range test. Graphs were generated using Graph Pad Prism Software (v.6.05), Inc.2015.

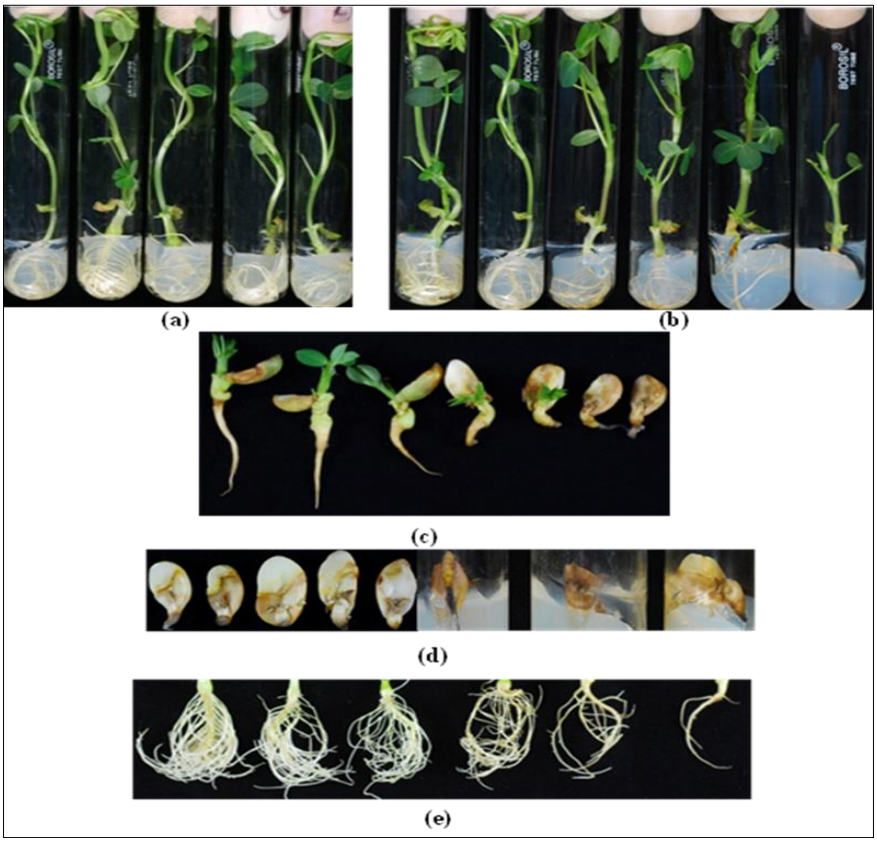


Fig 2: Effect of hygromycin on germination of peanut seedling on MS medium containing
(a) Controls without hygromycin; (b) Hygromycin at 1-6 mg/l (from left to right) (c) Hygromycin at 7-20 mg/l (from left to right) (d) Hygromycin at 20 & 40 mg/l (from left to right) (e) Root formation on hygromycin at 0-5 mg/l (from left to right).

Assay of the effects of different concentrations of hygromycin on the growth of peanut embryos

After 15 d of culture of embryos on MS with different concentration of hygromycin, the observations were evident of the effect of increasing concentrations of hygromycin on embryo development. The changes in the embryo at different concentrations of hygromycin were: (a) at 5 mg/l, hypocotyl-radical region was swollen, primary root was elongated and plumule turned green; (b) at 10mg/l, no elongation of radical was noticed, color changed to whitish brown; (c) at 15mg/l, no elongation of radical, but both the radical and plumule turned brown and necrotic; (d) at 20-35mg/l, no change in the explants and subsequently embryos turned black. The development of seedlings was suppressed with the raising hygromycin concentrations. In controls, plumule and hypocotyls were green, primary root was elongated with lateral roots (Fig. 3a).

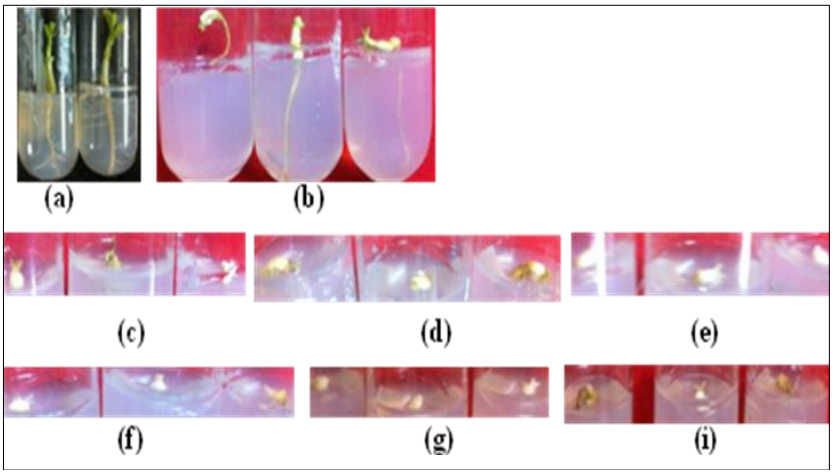


Fig 3: Effect of hygromycin on germination of embryo on MS medium containing (a) Control on 0 mg/l hygromycin (b) Hygromycin at 5 mg/l (c) Hygromycin at 10 mg/l (d) Hygromycin at 15 mg/l (e) Hygromycin at 20 mg/l (f) Hygromycin at 25 mg/l (g) Hygromycin at 30 mg/l (h) Hygromycin at 35 mg/l

Effects of hygromycin on the root of *Arachis hypogaea* seedling

Seeds of peanut (*Arachis hypogaea* L. var. JL-24) were cultured on MS or MS with 0-5 mg/l hygromycin, respectively and found that root growth was influenced drastically by elevated concentrations of hygromycin. The main root development on MS with hygromycin was very low when compared to the control on MS. Moreover, when the seedlings on MS with hygromycin was shifted to and cultured on MS without hygromycin, there was not much change in the main root and the lateral root development was ceased. With the increase in hygromycin concentrations, there was a decline in the root length and the number of lateral roots (Fig. 2e).

In the experiments to determine the effect of hygromycin, it was observed that, with an increase in hygromycin concentration, there was a drastic decline in the survival frequency of both the explants. The growth of embryonated cotyledons was partially inhibited till 15 mg/l. The elongation of the main root was significantly inhibited at concentrations greater than 10 mg/l and the formation of lateral root was completely suppressed on 7 mg/l.

Conclusion

The threshold concentration of hygromycin for the selection of transformants was optimized by culturing embryonated cotyledons and embryos of peanut on MS media containing different concentrations of hygromycin. After 15 days of observation, embryonated cotyledons survived up to 15 mg/l hygromycin. The explants were completely bleached at 20 mg/l. Explants showed necrosis and shoot induction diminished completely at 40 mg/l hygromycin. By this observation, 20 mg/l hygromycin was chosen as the threshold concentration for the selection of putative transformants. In this study, however, 20 mg/l hygromycin for embryonated cotyledons and 15 mg/l for embryos have been found to be the threshold levels and these concentrations were suppress the growth of peanut plants. High selection pressure may be harmful and inhibit the growth of the transgenics. Therefore, hygromycin at 20 mg/l for embryonated cotyledons and 15 mg/l for embryos could be used to select and screen hygromycin- resistant transgenic peanut plants after genetic transformation.

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