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Research Article
In vivo and *in vitro* Application of Colchicine on Germination and Shoot Proliferation in Soybean [*Glycine max* (L.) Merr.]

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Abstract
Background and Objective: Soybean growth and productivity remain adversely affected by various biotic and abiotic stress factors like pests and drought. This study tested *in vivo* and *in vitro* pretreatment of seeds with colchicine to establish a tissue culture-based protocol for soybean polyploidisation, with the benefit of potentially conferring inherent stress resistance in this crop. **Materials and Methods:** Seeds of 2 soybean cultivars viz. Peking and Dundee were pretreated with different concentrations of colchicine (0.0, 0.01, 0.05 and 0.1%) and sub-cultured for germination followed by *in vitro* shoot induction and plantlet regeneration. **Results:** The findings indicated that seed germination and shoot proliferation were inhibited by both *in vivo* and *in vitro* pretreatment of seeds with 0.05 and 0.1% colchicine, particular in Peking, compared to Dundee, followed by 0.01% used for the same cultivars. These effects were accompanied by severe tissue senescence and callus formation. **Conclusion:** *In vivo* pretreatment of soybean seeds with colchicine was more efficient and suitable for multiple shoot proliferation and regeneration under plant tissue culture conditions than *in vitro* application of colchicine for this purpose.

Key words: Colchicine, peking, dundee, germination, shoot proliferation

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Soybean, [*Glycine max* (L.) Merr.] belongs to the family Fabaceae, constituting a large number of varieties in which many are bred and developed for both subsistent and commercial farming worldwide. This crop serves as one of the main sources of high quality proteins (40%), lipids (20%), water-soluble carbohydrates (10%) and vitamins that are mostly contained within the seed endosperm[1,2]. A wide variety of fermented and unfermented soy-products have been explored, including its potential use for biodiesel manufacturing[3,4]. With the advent of these benefits, legume research has been directed toward improving seed quality and yield in order to continue the domestication of soybean. New genetic characteristics are required for improved growth and productivity, especially under changing adverse environmental conditions. Several breeding programs and mutagenic techniques have been reported[5,6], with a few varieties derived from these methods, including cowpea, faba. bean and pigeon pea cultivars[7]. However, there is no much genetic diversity that has been achieved to obtain new traits, creating genetic variability and supplementing conventional breeding techniques. For this purpose, the modification of soybeans through polyploidisation (i.e., mechanism of chromosome multiplication by inhibiting mitosis using colchicine) could also be used as an alternative for traditional breeding methods or genetic engineering. Ploidy manipulation with a chemical mutagen (for example, colchicine) is one of the most important techniques used to improve genetic diversity of plants, which confers inherent built-in resistance to various stress factors by modifying morphological and physiological characteristics[8]. However, the use of high levels of colchicine is fatal in plant tissues, because it is usually required in lower concentrations, which may be inadequate to induce successful polyploidisation[8]. According to the limited literature available, polyploidisation was never tested in soybean through *in vitro* plant tissue culture. Therefore, the main aim of the present study was to evaluate the effect of *in vivo* and *in vitro* pre-treatment of seeds with colchicine on germination and shoot proliferation in soybean.

MATERIALS AND METHODS

Plant material and decontamination: Soybean seeds cultivar Dundee and Peking were used, obtained from the Department of Biodiversity (Botany), at the University of Limpopo. The seeds were planted and self-pollinated for multiplication from September, 2018 to January, 2019. Seed decontamination was done by surface gas sterilisation with a mixture of hydrochloric acid and sodium hypochlorite as described by Mangena *et al.*[9], where harvested seeds were disinfected using liberated chlorine gas for 16 h at room temperature.

Chemical pre-treatment of seeds with colchicine: The disinfected soybean seeds were then pre-treated with colchicine at 0.0, 0.01, 0.05 and 0.1%. However, the decontaminated soybean seeds were weighed using a balance scale prior and post pre-treatment with colchicine. This was conducted to explicate the change in mass percentage of the seeds, which was calculated using Eq. 1, where, ΔSw stands for percentage change in 100-seed weight, W_i is the initial weight of seeds and W_f is the final weight after inhibition. *In vivo* pretreatment was carried out by imbibing the seed in a solution containing colchicine, meanwhile *in vitro* treatment was done on Murashige and Skoog[10] (MS) basal culture medium:

$$\Delta Sw = \frac{W_f - W_i}{W_i} \times 100 \quad (1)$$

For *in vivo* pre-treatment, soybean seeds were pre-soaked in a solution containing colchicine (0.01, 0.05 or 0.1%) by placing on a Gallenkamp orbital shaker (147 rpm) for 12 and 24 h, respectively, with gentle agitation in the dark. Seeds soaked in sterile distilled water were used as a control. Seeds were then briefly rinsed with autoclaved double-distilled water at the end of the incubation and sub-cultured on MS basal culture medium prepared according to Pierik[11] and then incubated for germination and seedling development. The final germination percentage was calculated as indicated below (Eq. 2), where, FG is the mean final germination percentage, G is the total number of germinated seeds and S is the total number of sub-cultured seeds. A total of 10 replicates/treatment with 3 soaked seeds for each culture vessel were prepared.

$$FG = \frac{G}{S} \times 100 \quad (2)$$

The *in vitro* pre-treatment was evaluated by inoculating soybean seeds on MS basal culture medium supplemented with varied colchicine concentrations as indicated above. The different colchicine amounts were pipette out from the colchicine stock solution, into the MS medium immediately after autoclaving at 121°C for 20 min. Soybean seeds used as a control were sub-cultured on colchicine free MS medium and all cultures were then incubated in a plant tissue culture

growth room for 10 days. Germination was also recorded daily and the mean final germination percentage was calculated using Eq. 2 as indicated on the section above. Seed pre-treatment with colchicine during germination was visually assessed and the mean number of swollen seeds, showing cracked seed coats was recorded after three days of incubation to ascertain the absorption of colchicine from the medium. All *in vitro* seed cultures were incubated in the dark, in a tissue culture growth room.

Shoot proliferation: For shoot proliferation, all colchicine pre-treated and non-pretreated seedlings were used to prepare double cotyledonary node explants used to establish shoot cultures. The explants were prepared by removing the root radical and primary shoots (epicotyls) and then sub-cultured on MS medium supplemented with 2.00 mg L^{-1} benzyl adenine (BA) (2 explants/culture vessel). All cultures were maintained in a growth room under 16 h photoperiod, 24±2°C temperature and 60 µmol m^{-2} sec^{-1} light intensity using cool white fluorescent tubes (Philips, Germany) for 2-7 weeks.

***In vitro* elongation and rooting:** All induced shoots were efficiently elongated and rooted on MS medium without plant growth regulators (PGRs). The well-developed shoots of about 3-5 cm were excised-off the explants and sub-cultured on hormone free medium for rooting. Rooted shoots were then transferred to sterile vermiculite for acclimatisation in a growth room at 24±2°C temperature, 120-200 µmol m^{-2} sec^{-1} white fluorescent light and 16 h photoperiod and then grown further in a greenhouse under natural conditions.

Growth conditions, data collection and analysis: All germination and shoot cultures were maintained in a tissue culture growth room under 50-60 µmol m^{-2} sec^{-1} light intensity, 24±2°C temperature and 16 h photoperiod. Visual observation of cultures showing shoot proliferation in terms of multiple shoot buds, shoot organogenesis, differentiation, number of shoots per explants, shoot length, callus formation, tissue senescence, root number and root length were recorded after 2 weeks of culture. Each treatment consisted of 10 replicates and the experiments were repeated at least thrice at an interval of 5 days. Data were analysed using analysis of variance (ANOVA) and Duncan's multiple test was used to determine the significant effect of the means.

RESULTS

***In vivo* colchicine pre-treatment of seeds on germination and shoot proliferation:** The effect of colchicine on germination, shoot proliferation and regeneration was evaluated by pre-treating soybean seeds *in vivo*, in a solution containing 0.0, 0.1, 0.05 and 0.01% of colchicine. The results showed that, percentage germination and percentage seed weight of the pre-treated seeds were affected by the presence of colchicine during imbibition (Table 1, Fig. 1). The findings exhibited significant differences in seed weights between the control and seeds soaked in colchicine solutions, respectively (Fig. 1). Seed weights appeared to decrease with the increase in colchicine levels, but also increased with the length of the imbibitions period (Fig. 1a-f). Further observations indicated that, germination percentage depended upon the amount of colchicine used. A significant decrease in percentage germination was noticed under increasing levels, especially at 0.1% followed by 0.05% of colchicine with 50.0 and 66.5% obtained in Peking and Dundee, respectively. Generally, seed germination was decreased due to colchicine in the cultivars used.

Table 1: Effect of colchicine on germination and shoot proliferation using cotyledonary explants developed from *in vivo* pre-treated seeds

Treatment period with colchicine	Final germination (%)	Explants with shoot buds (%)	Mean shoot number±SE	Shoot proliferation (%)	Callus formation
Dundee 12 h					
0.1	66.8^{e}	80.0^{d}	2.03±0.27^{e}	0.00^{d}	++
0.05	77.3^{d}	100.0^{a}	2.70±0.13^{d}	10.00^{c}	+
0.01	89.9^{c}	100.0^{a}	3.52±0.26^{b}	30.00^{b}	-
Control	100.0^{a}	100.0^{a}	6.04±0.13^{a}	90.50^{a}	-
Peking 24 h					
0.1	50.0^{f}	60.0^{e}	0.00±0.00^{g}	0.00^{d}	++
0.05	90.5^{b}	90.0^{c}	1.33±0.29^{f}	0.00^{d}	+
0.01	89.9^{c}	95.0^{b}	4.72±0.22^{c}	10.00^{c}	+
Control	100.0^{a}	100.0^{a}	8.06±0.11^{a}	90.50^{a}	-

Data are presented as the mean of the 10 replicates repeated thrice, percentage of shoot proliferation was evaluated as the total number of explants inducing more than 3 adventitious shoots over total number of explants sub-cultured for multiple shoot proliferation, +: Present of friable callus, -: Absent of callus, ++: Present of brown-black compact callus, values with similar letters are not significant at 5% confidence level using Duncan's test

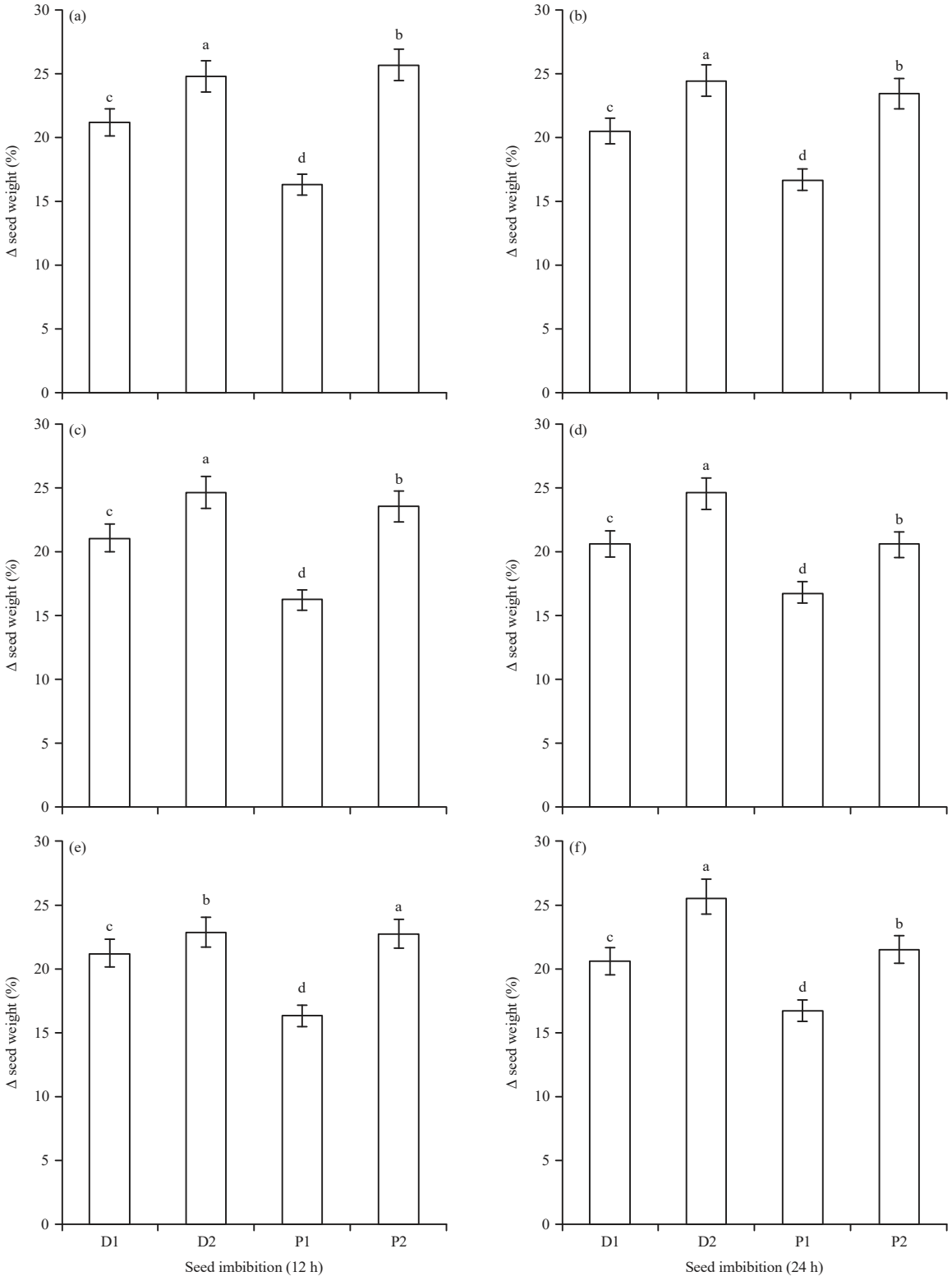

Fig. 1(a-f): Percentage change in seed weights after 12 and 24 h of *in vivo* seed imbibition of soybean seeds in a solution containing (a-b) 0.1, (c-d) 0.05 and (e-f) 0.01% colchicine

D1: Colchicine pretreated seeds of cultivar Dundee, P1: Colchicine treated seeds of cultivar Peking, D2, P2: Control

Table 2: Effect of colchicine on germination and shoot proliferation using cotyledonary explants developed from *in vitro* pretreated seeds

Treatment with colchicine	Final germination (%)	Explants with shoot buds (%)	Mean shoot number	Shoot proliferation (%)	Callus formation
Dundee					
0.1	49.6^{e}	60.0^{f}	1.03±0.27^{g}	0.00^{h}	-
0.05	53.8^{d}	75.0^{d}	2.30±0.14^{e}	15.0^{f}	+
0.01	60.1^{c}	80.0^{c}	2.74±0.25^{d}	45.0^{d}	+
Control	89.9^{b}	100.0^{a}	6.05±0.12^{b}	90.0^{a}	-
Peking					
0.1	45.9^{f}	55.0^{g}	1.88±0.22^{f}	10.0^{g}	++
0.05	42.9^{g}	60.0^{f}	1.93±0.19^{f}	30.0^{e}	+
0.01	50.3^{e}	65.0^{e}	3.44±0.22^{c}	55.0^{c}	+
Control	94.2^{a}	90.0^{b}	7.52±0.18^{a}	85.5^{b}	-

Data are presented as the mean of the 10 replicates repeated thrice, percentage of shoot proliferation was evaluated as the total number of explants inducing more than 3 adventitious shoots over total number of explants sub-cultured for multiple shoot proliferation, +: Present of friable callus, -: Absent of callus, ++: Present of brown-black compact callus, values with similar letters are not significant at 5% confidence level using Duncan's test

Based on the number of explants with buds, mean shoot number and percentage shoot proliferation (Table 1), colchicine also had a negative effect on multiple shoot induction and regeneration. Results showed that, the highest mean number of shoots was recorded in the control (6.04 mean shoot No. in Dundee and 8.06 in Peking) followed by explants derived from seeds pre-treated with 0.01% colchicine (3.52 mean shoot No. in Dundee and 4.72 in Peking). The lowest mean number of multiple shoots was produced by explants derived from seeds pre-treated with 0.1% colchicine (2.03 mean shoot no. in Dundee and 0.0 in Peking). These are exemplified in Fig. 2a-c, respectively, while *in vitro* elongation shown is Fig. 2d, roots in 2e and soybean shots in 2f. The colchicine concentrations used and soaking duration, especially for 24 h, were probably not appropriate, as a result, multiple adventitious shoots were not induced. After 2 weeks, more than 70% of the explants pre-treated with 0.1 and 0.05% of colchicine started producing compacted callus cells, mostly at the cut hypocotyl bases (Fig. 2d), as well as at the cotyledonary junctions. This probably inhibited shoot buds initiation and further development by blocking nutrient absorption. A high shoot proliferation percentage and less callus formation were observed in 0.01% than any other amount of colchicine used for pre-treatment. No callus formation was recorded in shoot cultures used as a control, which exhibited significantly higher number of multiple shoots (Fig. 2c).

Effect of *in vitro* pretreatment of seeds on shoot proliferation: The effect of colchicine pre-treatment was also assessed *in vitro* on MS medium. Data showed that, seed cultures did not exhibit typical germination patterns (Fig. 3), particularly in terms of the change in seed weights, which appeared gradually increased compared to the control, based on visual assessment. Water absorption capacity of the seeds was somewhat affected, either by the seed coats or by medium composition. Sub-culturing the seeds on MS medium containing colchicine (0.1, 0.05 and 0.01%) caused a fourfold decrease in the rate of seed germination when compared to *in vivo* pre-treatment. During the *in vitro* treatment of seeds with 0.1% colchicine, mean germination did not even reach 50% (Table 2). However, the mean germination percentage of the seeds that served as a control had already double within 5 days of incubation, compared to all seeds germinated on medium containing colchicine. Majority of the seeds died upon subculturing for 10 days on MS medium supplemented with colchicine. But, those that germinated, especially in MS medium containing a lesser amount of colchicine (0.01%) showed a dramatic increase in the growth of roots, hypocotyl stems and epicotyl shoots. Seedlings obtained on medium containing 0.01 and 0.05% colchicine generally presented thicker stems, thicker roots and longer branching roots compared to those that did not contain colchicine of the controls.

Only the explants developed from seeds pre-treated with 0.01% colchicine had better shoot proliferation responses than all treated cultures. Variations were generally showed in terms of the number of explants inducing shoot buds, mean number of shoots and mean percentage of shoot proliferation (Table 2). Soybean cultivar Peking induced the highest mean number of shoots than cultivar Dundee, demonstrating this throughout the experiments. Results also showed that, higher concentration of colchicine (0.1%) was detrimental to explants and their shoot response (Fig. 2d), compared to lower concentration such as 0.01% as shown after *in vivo* pre-treatment of seeds (Fig. 2a). A unique observation, however, was made on cotyledonary node explants derived from seeds pre-treated with 0.05% of colchicine. About 70% of these explants managed to successfully induce the two axillary shoots from pre-existing axillary meristems on the

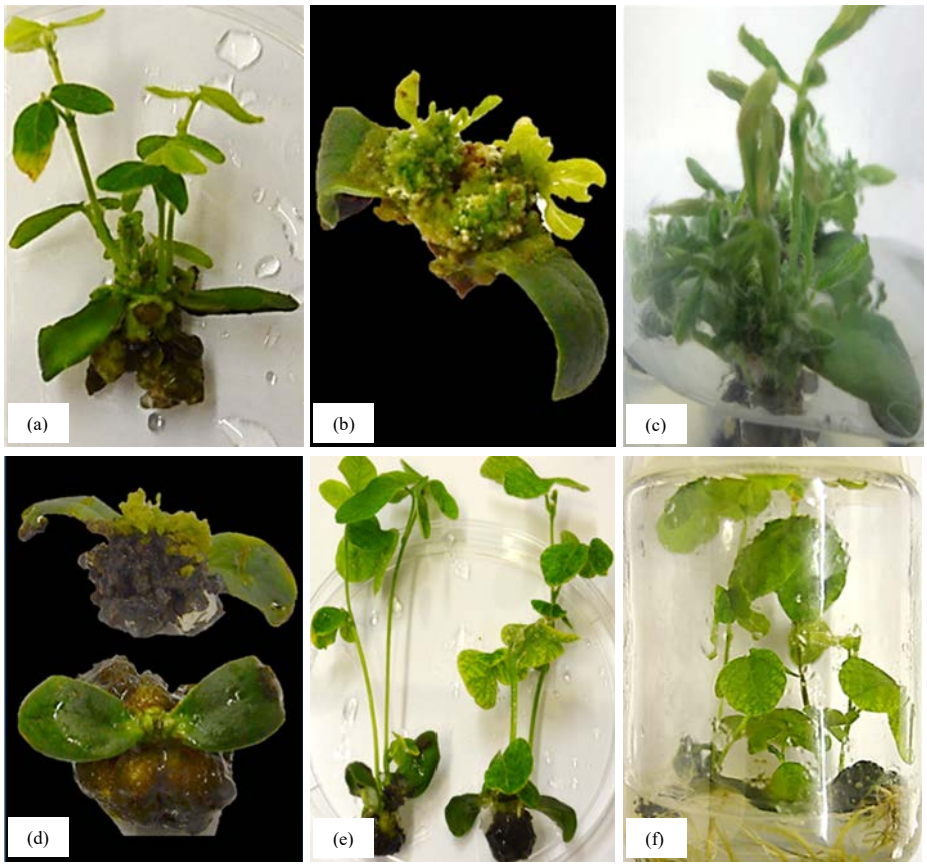

Fig. 2(a-f): Shoot proliferation and regeneration response of cotyledonary node explants derived from seeds pretreated with different levels of colchicine. Multiple shoots induced on explants pretreated with (a) 0.01% colchicine, (b) Shoot buds formed on explants treated with 0.05% colchicine, (c) Control without colchicine pretreatment, (d) Cotyledonary nodes pretreated with 0.1% colchicine showing failure to induce shoots, (e) Examples of *in vitro* elongated and (f) Rooted soybean shoots

cotyledonary junctions. The cultures mostly induced axillary shoots than multiple adventitious shoots compared to what was obtained in 0.01% colchicine (Fig. 2a) and the control (Fig. 2c). Furthermore, the induced axillary shoots were rapidly elongated (Fig. 2e) and all of the shoots formed were easily and directly rooted without any use of plant growth regulators or changing the medium compositions and transferring into a new fresh culture medium (Fig. 2f).

***In vitro* elongation and rooting:** The overall responses of successfully *in vitro* elongated and rooted shoots is summarised in Table 3. Hormone free MS medium was efficiently used for this purpose. However, variations in percentage elongation between adventitious shoots formed from lower concentrations of colchicine (0.05 and 0.01%) to increased concentration (0.1%) were observed. Maximum mean elongation percentage of 60 and 75.5% were obtained in Dundee and Peking, respectively. All cultures derived from 0.05% colchicine pretreatment recorded the highest average shoot lengths of 6.2 cm for Dundee and 7.5 cm in Peking (Table 3). A few number of shoots sub-cultured for elongation and rooting percentages were recorded in both cultivars for 0.1% colchicine pre-treatment. All elongated shoots were efficiently rooted on the same hormone free MS medium (Table 3). Moreover, the results did not show any differences between or within the treatments for average root lengths and survival frequency of the rooted plantlets. Although, differences in the estimated root lengths were recorded, this could not be attributed to the colchicine concentration since the controls also gave similar results (Table 3). All cultures showed a high rate of survival, above 70% for all regenerated soybean plantlets for both cultivar Dundee and Peking.

Table 3: Overall effects of colchicine on elongation, the length of shoots, rooting and acclimatisation of plantlets regenerated under both *in vivo* and *in vitro*

Treatment with colchicine	Elongation (%)	Shoot length (cm)	Rooting (%)	Root length (cm)	Plantlet survival rate (%)
Dundee					
0.1	20.0^{h}	4.5±0.25^{f}	100^{a}	5.8±0.66^{f}	95.0^{b}
0.05	60.0^{e}	6.2±0.61^{bc}	100^{a}	6.5±0.50^{e}	80.0^{d}
0.01	40.5^{f}	5.4±0.22^{e}	100^{a}	5.4±0.46^{g}	100.0^{a}
Control	90.0^{b}	6.0±0.50^{cb}	100^{a}	5.8±0.66^{f}	100.0^{a}
Peking					
0.1	30.0^{g}	6.1±0.48^{bc}	100^{a}	9.3±0.60^{a}	80.0^{d}
0.05	75.5^{c}	4.4±0.39^{g}	100^{a}	8.2±0.56^{d}	70.0^{e}
0.01	65.0^{d}	7.5±0.55^{a}	100^{a}	8.5±0.70^{bc}	90.0^{c}
Control	100.0^{a}	6.0±0.60^{cb}	100^{a}	8.4±0.54^{cb}	90.0^{c}

Data are presented as the mean of the 10 replicates repeated thrice, percentage of elongation refers to total number of explants inducing shoots sub-cultured for elongation, percentage of rooting is the total mean percentage of the number of shoots inducing roots, survival rate (%) refers to the total number of rooted plantlets showing successful acclimatisation and growth under natural conditions, mean values with similar letters are not significantly different according to two-tail excel t-test at 5% confidence level

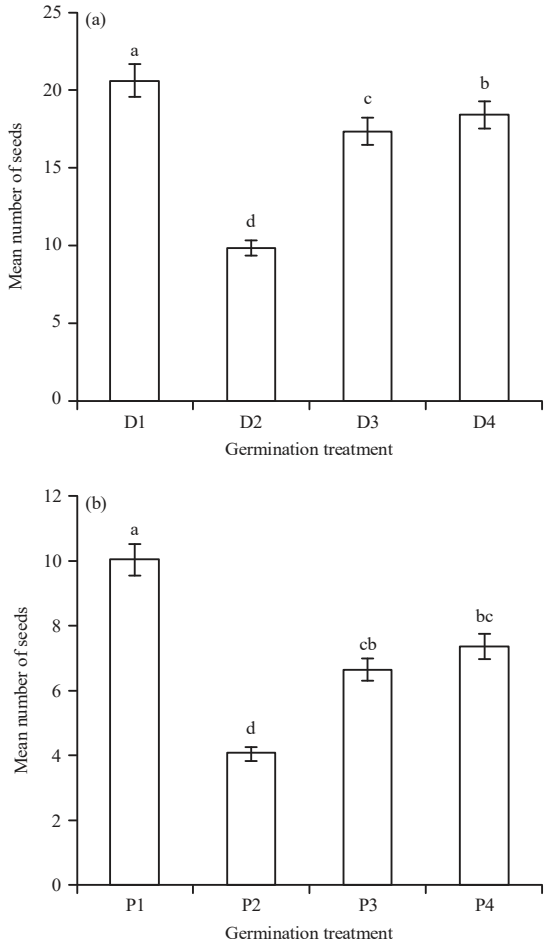

Fig. 3(a-b): Visual assessment of soybean seeds following incubation of seeds subcultured on MS culture medium containing colchicine in cultivar (a) Dundee (D) and (b) Peking (P)

D1, P1: Controls, D2: 0.1%, D3: 0.05%, D4: 0.01%, P2: 0.1%, P3: 0.05%, P4: 0.01% indicate varying levels of colchicine treatments

DISCUSSION

The *in vivo* soybean seed pre-treatment with colchicine produced different percentages of germinated seeds and seed weights. In addition, the varied responses were greatly influenced by the duration of imbibition and the genotype. These results implied that soaking seeds in a colchicine solution could delay imbibition and germination. Furthermore, it was clear that, higher concentrations of colchicine (0.05 and 0.1%) delayed germination, especially of Peking seeds which took longer to break the seed coats. Although, Mangena and Mokwala[12] suggested that Peking seeds possess hardier black seed coats, which was reported to cause gradual seed imbibition.

Generally, seed treatment with colchicine affected seed germination under both *in vivo* and *in vitro* conditions, by decreasing the speed and uniformity of germination. It was also clear that culturing the seeds on MS medium supplemented with 0.05 and 0.1% of colchicine did not increase the rate of germination. Evidently, 0.1% colchicine had dramatic effects on germination, particularly in Peking compared to Dundee for the entire experiment. This was again due to the level of colchicine used and probably as a result of the nature of hardier seed coats in Peking and the genotypic difference as indicated by Cheng and Hadley[13]. High colchicine concentrations and seed coat textures also caused adverse effects on the germination of *S. coccinea* and *V. cracca* as reported by Kobayashi *et al.*[14] and Munzbergova[15].

The *in vivo* and *in vitro* shoot proliferation results in Table 1 and 2 indicated that colchicine levels (0.05 and 0.1%) inhibited shoot formation in comparison with the untreated controls. The poor shoot induction was also influenced by the duration of seed imbibition, particularly the 24 h used. These

effects were previously reported in *Colophospermum mopane* using 0.05 and 0.1% of colchicine for 48 h Rabuluza *et al*.[16]. This study also reported 0% survival rate of *C. mopane* seedlings obtained from seeds imbibed in 1% colchicine for 48 and 96 h. Similar findings were also reported by Blakesley *et al*.[17] and Wu and Mooney[18] in *Acacia dealbata* and *Acacia mangium*, as well as *Citrus* spp., respectively. Analysis of explants derived from seeds germinated on MS medium containing colchicine for shoot proliferation also showed similar results. It was clear that; shoot proliferation was severely affected by prolonged exposure of seeds to colchicine, contributing to the unresponsiveness on shoot induction MS medium and subsequent deaths of explants. Similar findings were made by Głowacka *et al*.[19], indicated that, colchicine significantly influenced the effectiveness of shoot regeneration in 2 *Miscanthus* species. Generally, colchicine concentration (0.01%) had effected shoot proliferation, leading to successfully *in vitro* regenerated soybean plantlets for seeds pre-treated *in vivo*. The putative regenerated plants had significantly larger leaves, longer stems, many roots, overall vigorous growth and highly significant rates of survival, comparable to the control soybean plants.

CONCLUSION

This is the first reported study in soybean, indicating colchicine as an effective agent for potential polyploidy induction in this species. The *in vivo* pre-treated seeds of soybean could be suitably used to derive colchicine treated (polyploidised) seedlings in which further multiple shoots can be proliferated and regenerated under *in vitro* plant tissue culture conditions than pre-treatment *in vitro*. Both durations of imbibition, 12 and 24 h, could be preferable, compared to *in vitro* pre-treatment of soybean seeds, for polyploidisation purposes.

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SIGNIFICANCE STATEMENT

This study reported on the effect that soybean seed pre-treatment with colchicine has on *in vitro* germination and shoot multiplication. Results on the 3 varying levels of colchicine used (0.0, 0.01, 0.05 and 0.1%), including control, reveals possible establishment of an *in vitro* protocol that can be used to improve morphological and physiological characteristics of soybean, using this polyploidising agent. According to literature, there are very limited studies done on this topic to date, especially the use of *in vitro* plant tissue culture and there are no reports on protocols used for *in vitro* development of soybean polyploids.

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