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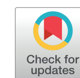
Research article

Analysis of correlation between seed vigour, germination and multiple shoot induction in soybean (*Glycine max* L. Merr.)



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ABSTRACT

The physical and physiological roles of seed properties are often neglected during plant tissue culture. These properties determine the level of activity and performance of seeds, which is commonly known as seed vigour. This paper reports on the role of seed vigour on seed germination and shoot induction using cotyledonary node explants. For this, explants prepared from soybean seedlings established using seeds stored under ambient conditions for different durations (0, 3, 6 and 9-months) were used. The findings indicated that seed germination was highly instantaneous after harvest and began to decrease as seed storage was prolonged for 3, 6 and 9-months, respectively. Similar observations were made during shoot induction. Generally, the analysis revealed a positive relationship between seed vigour, germination and multiple shoot initiation as indicated by the Pearson's correlation coefficient reported. According to the findings, seed vigour could serve as a major obstacle to efficient germination and shoot proliferation for subsequent *in vitro* plant regeneration.

1. Introduction

Seed germination is considered as one of the most critical stages for successful seedling establishment, efficient plant growth and development. Successful germination depends upon a number of factors that include the moisture content of the seeds, and growth conditions such as humidity as well as temperature (Bairu et al., 2009; Finch-Savage and Bassel, 2016; Selvi and Saraswathy, 2017). However, plant tissue culture protocols that rely upon germination serve as some of the most important tools used to provide strategies for growth improvement in cereal and legume crops. These techniques are inherently dependent on the physical and physiological properties of tissues targeted for use as explants/explant source, such as immature or mature seeds, cotyledonary nodes and hypocotyl nodes. Several studies have shown that the use of good quality seeds as explant source could improve plant regeneration (Zia et al., 2010; Singh et al., 2016; Raza et al., 2017; Wijewardana et al., 2019). In order to improve soybean growth and productivity, especially under biotic and abiotic stress conditions, numerous researchers have explored the propagation of soybean plants via tissue culture, coupled with genetic improvement to develop new varieties with enhanced vegetative and yield characteristics. These techniques may allow precise and controlled addition of new genes to the genome of targeted plant species, conferring traits such as fungal resistance, insect-pest resistance and improved tolerance to adverse environmental constraints. Amongst some of the severe abiotic stress factors, drought and salinity are considered the most damaging and growth limiting factors in soybean growth and productivity. However, the improvement of soybeans through *in vitro*-based plant regeneration and genetic improvement still occurs at a very low rate and most of the efficiently applied protocols are genotype specific, with numerous varieties remaining highly recalcitrant (Olhoft and Somers, 2001; Paz et al., 2006; Phat et al., 2015). While these challenges persist, many researchers believe that the increased efficiency in regeneration protocols hold the key to a successful development of a routinely applicable, effective and genotype-independent regeneration protocol. The most rapid and efficacious shoot induction and regeneration system that bypasses *in vitro* propagation barriers is highly required. While some researchers continue to investigate and optimise such tissue culture procedures, it will also be interesting to observe how seed vigour influences the germinability of seeds and the viability of explants developed from such germinated seeds.

Many studies identified moisture retention capacity of seeds, germination rates, uniformity and growth as the main components of seed vigour, that are also negatively influenced by seed storage conditions (Egli et al., 1990; Finch-Savage and Bassel, 2016; Ebone et al., 2020;

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Meneguzzo et al., 2021). These components are said to play a key role in germination and seedling development. Meneguzzo et al. (2021) reported differentiation of seedling growth and development as a result of vigour levels of seed lots in soybean cultivar 5855 RSF IPRO. High emergence speed and uniformity index were also reported by Ebone et al. (2020) from soybean seeds with different vigour levels (89, 57, 47 and 43%). This phenomenon was defined by de Venter (2008) and Marcos-Filho (2015) as an index of the extent of physiological/biochemical deterioration and mechanical integrity of the seed, which have the ability to negatively and/or positively influence the performance of seeds under a wide range of environmental conditions. This definition implies that seed vigour refers to seed properties with the potential for rapid, uniform emergence and development of healthy and vigorous quality seedlings. These seedlings are required during plant tissue culture to develop cotyledonary explants used for *in vitro* shoot regeneration and subsequent genetic transformation. Nonetheless, too little to none work have been done to evaluate the correlation between seed vigour and the stages involved during plant regeneration. This paper, attempts to analyse and generate insights on this side of the story. The study evaluated the effects of seed vigour on *in vitro* germination and shoot induction using six cultivars of soybean in order to properly correlate these tissue culture stages with seed vigour. Furthermore, similar compositions of Murashige and Skoog (MS) basal culture medium, type, the amount of plant growth regulators, and growth conditions were kept unchanged with an intention to assess the correlation effectively.

2. Materials and methods

2.1. Plant material and culture conditions

Mature seeds of six cultivars of soybean (*Glycine max* L. Merr.), viz Dundee, LS677, LS678, Peking, TGx-1740-2F and TGx-1835-10E, were used in this study. Seeds were obtained from the Department of Biodiversity and from the Department of Plant Production, Soil Science and Agricultural Engineering, at the University of Limpopo. The soybean seeds were replanted at Amaloba Nursery at the University of Limpopo, Turfloop campus, South Africa, and freshly harvested in the months of February–March 2019. Fresh seed materials were harvested at maturity from each cultivar and stored in brown paper bags for further analysis. The harvested seeds were counted into sets of 100, separated into multiple batches and weighed before they are stored under ambient conditions until used. Seed vigour index was determined using formula (1) below, where V is the vigour/viability index, K_i is the initial 100 seed weight immediately after harvest, ΔSw is the change in seed weight measured after the duration of storage and *P* is the period of storage in days. Data relating to changes in seed weights and vigour index was used to evaluate the performance of soybean seeds following seed storage under ambient conditions.

$$Seed\ vigour\ (V) = Ki - \left(\frac{1}{\Delta Sw}\right)P \quad (1)$$

2.2. Decontamination and germination of seeds

Soybean seeds were disinfected using chlorine gas according to Mangena et al. (2015) before they are used for *in vitro* germination. Petri dishes containing seed samples prewashed with soapy tap water, rinsed several times with sterile distilled water and air-dried, were placed in a desiccator jar in the fume hood. A 250 ml beaker containing 100 ml of sodium hypochlorite was placed in the jar with the seeds and 3.5 ml concentrated hydrochloric acid was carefully added into the beaker with bleach. The jar was immediately closed and the seeds were surface sterilised with the liberated chlorine gas for 16-hours. *In vitro* germination of seeds was done on Murashige and Skoog (MS) medium containing full strength MS salts, 3% sucrose and 0.25% gelrite at pH 5.8. A predetermined amount of 8.877 μM of benzyladenine (BA) was added into the medium. The medium was distributed into 150 ml baby jar culture vessels before being sterilised by autoclaving at 121°C for 20 min, and 3 sterilised seeds per culture vessel were inoculated on the MS culture medium. Seed cultures were maintained in a growth room at 25 ± 2°C under a 16-hour photoperiod for 10 days. Light was provided by white phillips fluorescent light bulbs at about 50–60 $\mu molm^{-2}s^{-2}$. Seed germination was defined as the protrusion of root radicle and emergence of the epicotyl. The number of germinated seeds was then recorded on a daily basis, with the first and final counts were recorded after 24-hours and 10-days of incubation, respectively. A complete randomised research design was used throughout, and 15 replicates containing three seeds each were prepared per cultivar. Experiments for germination of all cultivars were repeated at least thrice, and the first germination test was conducted immediately after harvesting (0) and consecutively after 3, 6 and 9 months under similar conditions.

2.3. Explant preparation and subculture for shoot induction

Double cotyledonary node explants with axillary meristematic tissue were tested for shoot multiplication. The explants were prepared by removing the epicotyls and cutting off the hypocotyls 4–6 mm beneath the cotyledonous leaves. The methodology used for explant preparation was the same as the procedure described by Mangena et al. (2015) and Mangena and Mokwala (2019). A preliminary screening of different concentrations of cytokinins was conducted and according to the results, a preferred amount of 8.877 μM BA was selected for *in vitro* multiple shoot induction. The prepared cotyledonary node explants were subcultured on MS culture medium prepared as indicated for germination, and supplemented with 8.877 μM BA. Shoot cultures were incubated for more than 3 weeks under plant tissue culture conditions similar to those used for seed germination. The experiment was repeated thrice with 15 replicates containing two explants per culture vessel. Morphogenic patterns of shoot development were compared among the shoot cultures established from cotyledonary explants derived from seed lots stored for 0, 3, 6 and 9 months as indicated previously.

2.4. In vitro elongation and rooting

The induced shoots were excised from the explants and subcultured on hormone free medium for shoot elongation and rooting. This stage took about 7 weeks for efficient elongation and rooting to take place. The culture medium used was half-strength MS salts, supplemented with 3% sucrose and 0.25% gelrite. The pH was adjusted to 5.8 with the addition of 0.1 N NaOH or HCL before the addition of a solidifying agent. The culture medium was distributed into 150 ml baby jar culture vessels and autoclaved for 20 min as indicated for germination and shoot induction media preparation. The number of replicates were randomly prepared based on the number of induced shoots requiring elongation and rooting, but each contained at least 2 to 3 subcultured shoots for *in vitro* elongation and rooting per culture vessel.

2.5. Acclimatisation of regenerated plantlets

Plantlets acclimatisation was randomly done per week based on the established plantlets that required hardening under *ex vitro* growth conditions. The frequency of rooting was assessed weekly and based on this, rooted shoots with at least 3 or more adventitious roots of about 4–5 cm in length were removed from the rooting medium. Rooted shoots were first washed with sterile distilled water to remove the gelling agent and transferred onto sterile moistened vermiculite for further growth. The plantlets were covered with transparent plastic bags, which was gradually removed to facilitate hardening by reducing excessive water loss.

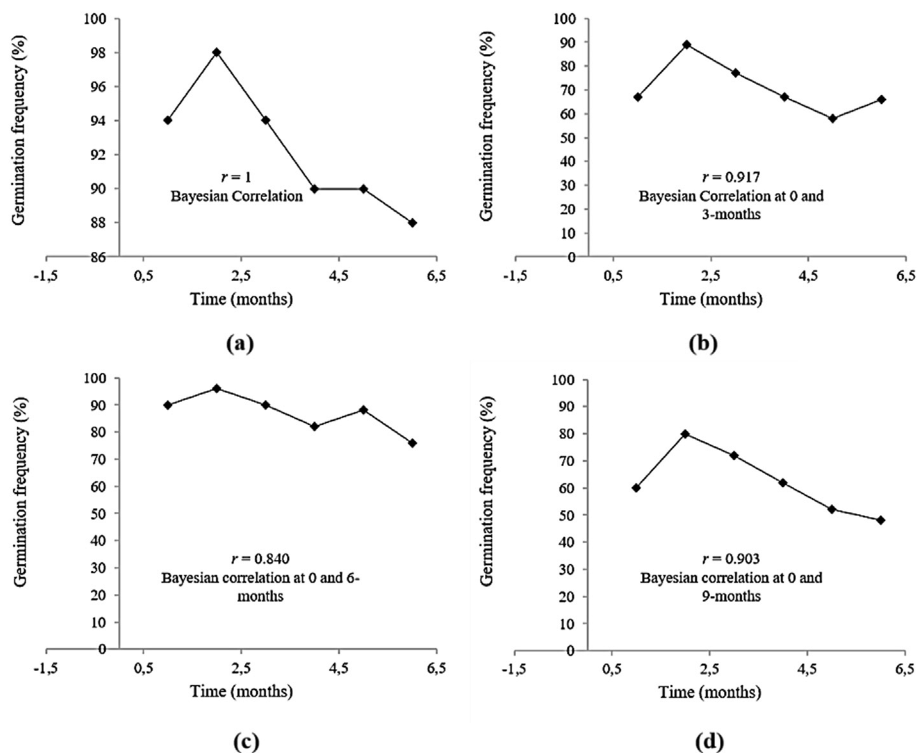

Figure 1. *In vitro* soybean germination trends versus the duration of storage of seeds under ambient conditions for 0 (a), 3 (b), 6 (c) and 9 (d).

Table 1. Statistical data calculated from the mean germination percentages following seed storage under ambient conditions.

	A	B	C	D
Std. Error of Mean	1.498	2.863	4.417	4.910
Median	92.000	89.000	67.000	61.000
Std. Deviation	1.498	2.863	4.417	4.910
Variance	13,467	49.200	117.067	144.667
Mean Difference	92.333	87.000	70.666	62.333
Sig. (2-tailed)	**.00021***	**.00074**	**.00017***	**.00011**

Note: A-refers to 0-month, immediately after harvest. Meanwhile D-, C- and B-denote to 9, 6 and 3-months of seed storage after harvest. Values with asterisk within a row are not significantly different at 95% confidence intervals.

2.6. Growth conditions

The cultures for *in vitro* seed germination and multiple shoot regeneration were maintained in a growth room at 25 ± 2°C temperature, 50–60 $\mu molm^{-2}s^{-1}$ light intensity and 16 h photoperiod. *In vitro* produced potted plantlets were kept under controlled environment at 25 ± 2°C with 120–200 $\mu molm^{-2}s^{-1}$ light for three to six weeks. Potted plants were watered daily with half-strength MS medium depending on vermiculite moisture content. After four to six weeks of gradually opening the transparent plastic bags to acclimatise regenerated plants, all hardened plants were moved to the glasshouse and kept under normal day length conditions.

2.7. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 26 software. Analysis of variance was applied and the significance between the means was compared using one-way ANOVA and the student t-test at the significance level of 0.05. Correlation graphs were plotted using

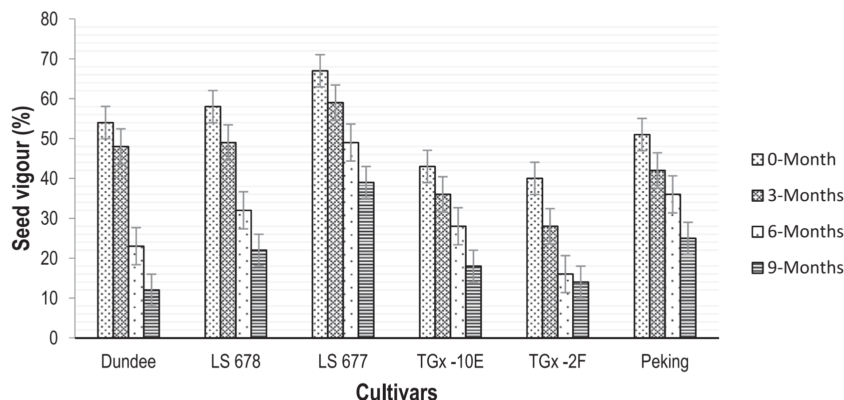

Figure 2. Percentage vigour indices determined using Eq. (1) during 0, 3, 6 and 9 months of seed storage under ambient conditions.

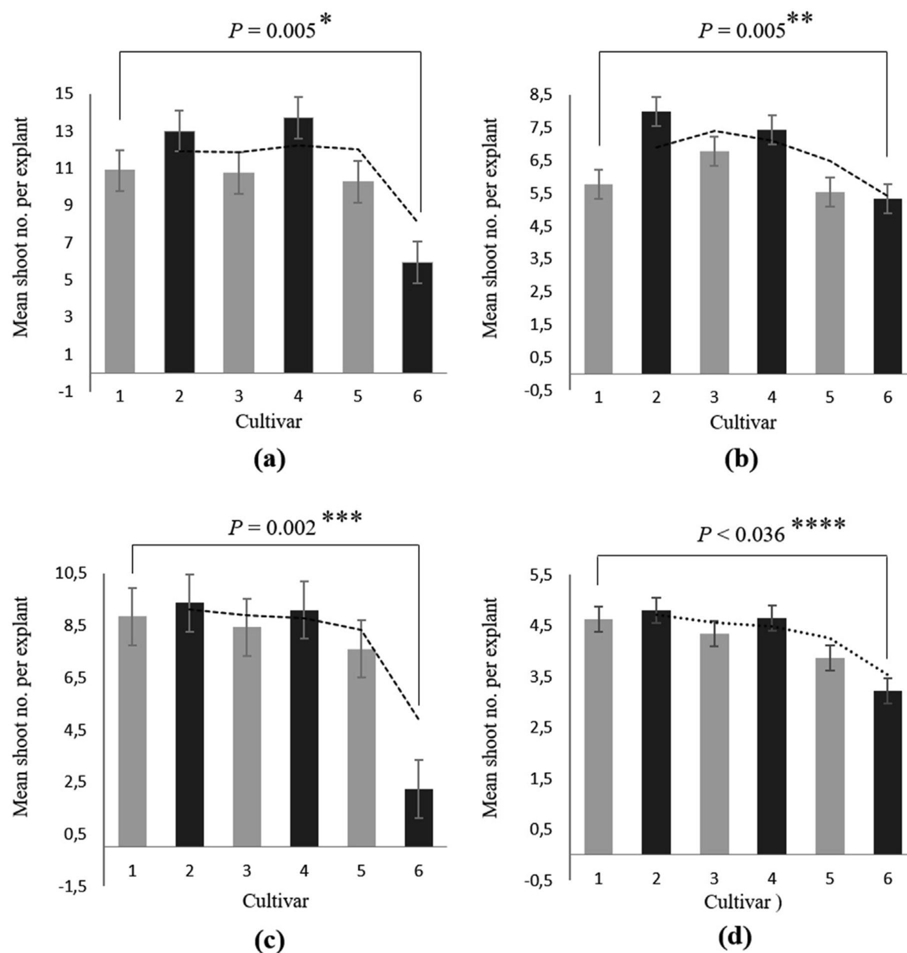

Figure 3. *In vitro* shoot induction trends in Dundee (1), LS677 (2), LS678 (3), Peking (4), TGx-1835-10E (5), and TGx-1740-2F (6) following seed storage under ambient conditions for 0 (a), 3 (b), 6 (c) and 9 (d) months.

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3. Results and discussion

3.1. The effect of seed vigour on germination

This study showed significant variations in the percentage of *in vitro* seed germinations which were observed among the different cultivars used and during the period of seed storage (Figure 1). The mean germination percentages for all cultivars (Dundee- 90%, LS677- 98%, LS678-96, Peking- 94%, TGx-1835-10E- 92% and TGx-1740-2F with 88%) were more than 80% during the first experiment which was performed immediately after harvesting. A decline in germination was then observed after using seeds stored for 3, 6 and 9-months under ambient conditions (Table 1). According to the results obtained in this study, seed vigour (1) influenced the frequency of germination in all cultivars, followed by the duration in which the seeds were stored. The decrease in seed vigour determined the level of activity, the performance of seeds during *in vitro* germination, and seedling development (Figure 2). As previously indicated, any losses of seed vigour calculated as indicated in formula (1) could be directly correlated with a reduction in seed germination and seedling development. This analysis on the germination frequency revealed a positive relationship between germination set against storage duration and seed vigour as indicated by the Pearson's correlation coefficient, demonstrated on Figure 1.

The observations made in this study and other previous studies (Mangena et al., 2015; Mangena and Mokwala, 2019) clearly showed that germination frequencies are more directly correlated with the age of seeds, quality and the genotype, instead of *in vitro* conditions. Hampton (1981) found no significant difference in the germination frequency and seed vigour of several wheat lines (*Triticum aestivum*) when grown on different soil conditions. This study indicated that there were no significant gains on germination achieved even when both the good and bad soil conditions were used. Their results relied upon the quality of wheat seeds used on field emergence trials reported in this experiment. In this study, soybean cultivar LS677 exhibited the highest mean germination percentage throughout the experiment as indicated above. This took place despite frequent and marginal reductions recorded. This could be as a result of the outcomes attributed to the genotype factor that determined the pace in which seed vigour was lost (Figure 2). This trend was similarly observed in cultivar LS678, and it was followed by seeds of Dundee and Peking. The germination responses obtained in this study are in line with the findings by Dubby and Dwivedi (1987), and recently by Singh et al. (2016). Both reports highlighted decreases in the performance of soybean plants based on their germination time, culture conditions and the genotype. The studies emphasised that seed storage had negative effects on seed vigour and germinability, where deterioration may also be genotype-dependent. With Singh et al. (2016) further stressing that seed storage is not permissible for more than a year, when it is aimed at maintaining high seed viability without causing any signs of deterioration. Although, *in vitro* germinations of most legume species, including soybean, could be far over 50% or lesser, limited data is available on the influence of vigour on seed germination rates under plant tissue culture conditions. Wijewardana et al. (2019) also reported germination figures of over 50–100 % from time-series germination data

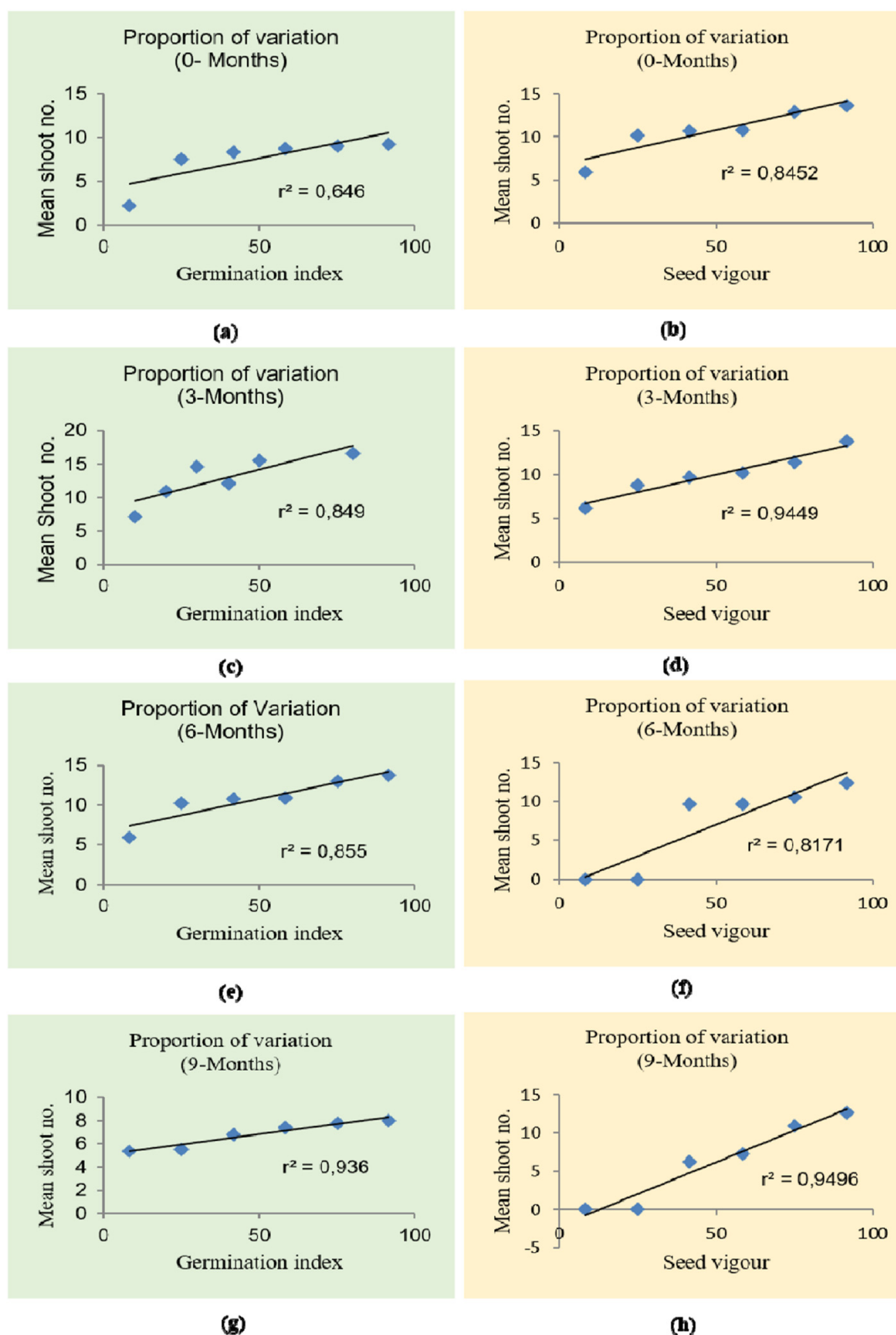

Figure 4. Regression analysis between shoot formation frequency per cultivar against both germination and seed vigour following 0 (a–b), 3 (c–d), 6 (e–f) and 9 (g–h) months of soybean seed storage under ambient conditions.

at different osmotic potentials in two soybean cultivars viz Asgrow AG5332 and Progeny P5333RY. In contrast, germination of these cultivars were said to primarily depend on culture conditions such as temperature and moisture than critical seed properties. Furthermore, the loss in seed vigour is related to the reductions in the ability of seeds to carry out both biochemical and physiological functions. For instance, changes in enzyme activity, membrane integrity and protein synthesis severely interferes with the seeds' ability to germinate.

3.2. *Effect of seed vigour on in vitro shoot induction*

The decrease in the frequency of shoot formation from seedlings derived explants reflected deterioration of seed vigour (Figure 2). It indicated further that the performance of seed germination is poor. Seed vigour and germination remain critically important for seedling establishment, explant source development and shoot induction. Poor germination normally results in a less acceptable quality of explants used in multiplication of shoots for efficient *in vitro* plant regeneration. A large number of adventitious shoots were obtained from the double cotyledonary explants derived from seedlings germinated using freshly harvested soybean seeds (Figure 3). Double cotyledonary nodal explants developed from these seeds and those initially stored for less than 3 months produced a reasonably high number of shoots compared to those stored for longer periods (6 and 9 months). The highest mean shoot numbers ± standard deviations of 13.73 ± 3.33, 13.00 ± 1.56, 10.73 ± 3.04, 10.87 ± 3.14, 10.27 ± 3.04 and 5.93 ± 2.34 were recorded for Peking, LS677, LS678, Dundee, TGx-1835-10E and TGx-1740-2F, respectively. Almost all cultivars, except TGx-1740-2F recorded over

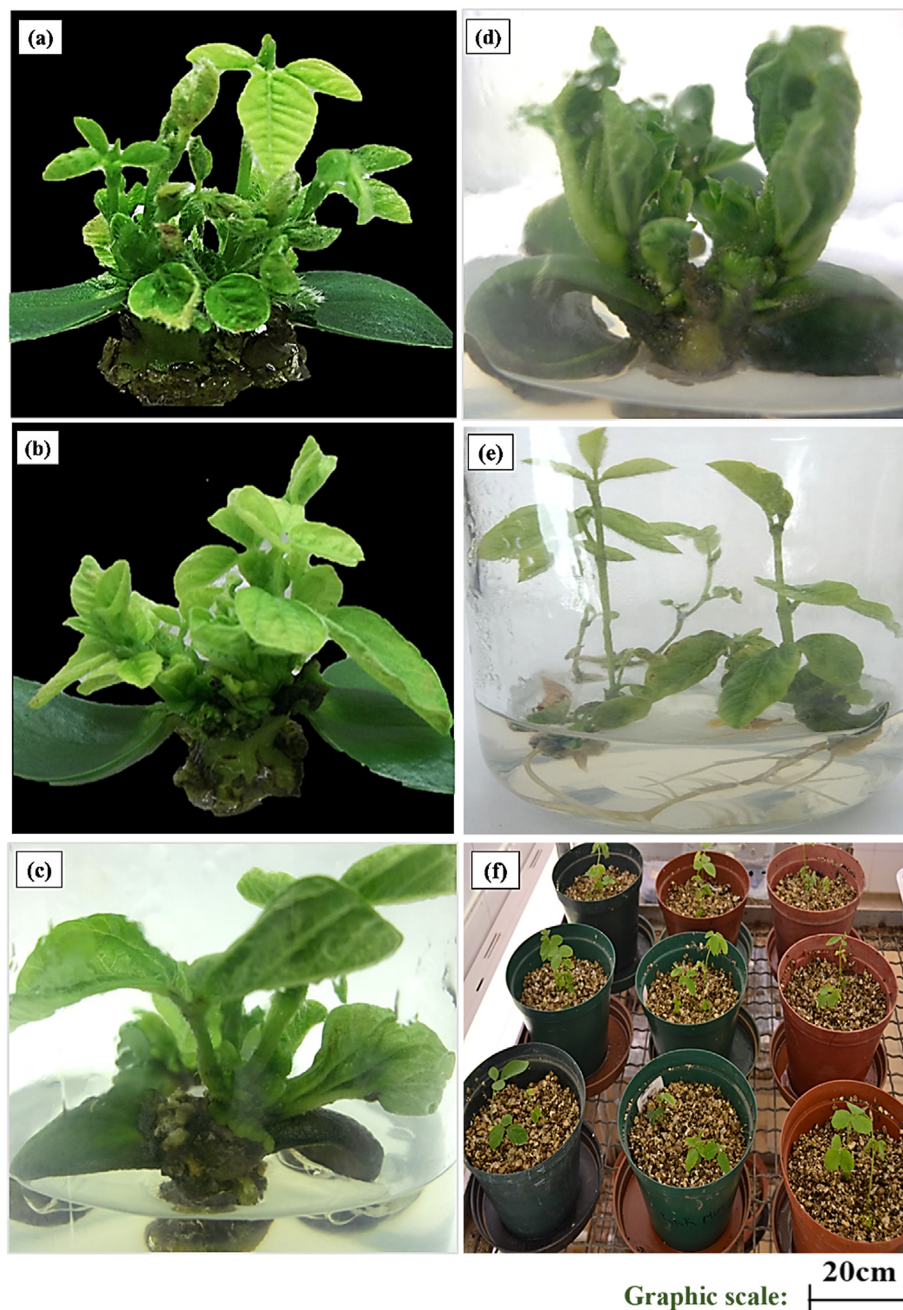

Figure 5. Shoot regeneration from cotyledonary nodal explants and acclimatisation of regenerated plantlets. Typical multiple shoot responses induced using explants derived from freshly harvested seeds (a) and seeds stored for 3 (b), 6 (c) and 9 (d) months under ambient conditions. *In vitro* elongation and rooting of regenerated shoots (e) and example of hardened regenerated plantlets (f).

10 shoots per explant initially before seed storage, but mean shoot number continuously showed a significant decline following the storage of seeds for 3, 6 and 9-months before shoot regeneration experiments. As indicated in our previous report (Mangena and Mokwala, 2019), overall low performance of TGx cultivars, particularly, TGx-1740-2F, may be attributed to their rapid loss of seed vigour during storage compared to other soybean cultivars (Figure 4).

MS culture medium and BA (8.877 μM) used, including the conditions of culture incubation were effective in initiating adventitious shoot formation and growth. Therefore, as indicated by the results obtained in this study (Figures 3, 4, and 5), shoot regeneration was strongly influenced by the quality of seeds used, probably followed by the genotype, as compared to the culture conditions. These observations suggested that a direct correlation between shoot formation and seed vigour exists, supported by the gradual reduction trends as illustrated in Figure 4 (a–d). Moreover, the findings further implied that, the declining seed germination previously observed may have also caused a decline in shoot induction as a positive correlation was found between shoot formation and germination as indicated in Figure 4 (a–h). As Daniel et al. (2003) emphasised, seeds deteriorate rapidly during storage and these effects are easily reflected by the decline in physiological responses, including seed vigour and seedling viability. Findings made in this study are in line with Daniel et al. (2003), Tang et al. (1999) and Simic et al. (2006) since positive correlations were observed, resulting from the loss of seed vigour during storage (Figure 4). The above studies also reported strong correlations between seed moisture content and vigour which similarly had subsequent negative effects on germination and seedling development. The results described above could be attributed to changes in the

physiological/biochemical status of the seeds that took place during seed storage since culture media and growth conditions were kept the same throughout the experiment. MS medium modified with plant growth regulators (cytokinins and auxins) have been routinely used to initiate cell division and differentiation during shoot organogenesis (Mangena and Mokwala, 2019; Dan and Reichert, 1998; Ali et al., 2021). But culture conditions in this case failed to influence shoot induction as illustrated by the difference in the number of shoots induced as exemplified in Figure 5 (a–d). It was clearly observed that shoot induction decreased with the increase in seed storage duration (Figures 2, 3 and 4). The manipulation of these conditions played a critical role for protocol optimisation, especially for efficient regeneration and tissue culture-based genetic improvement of recalcitrant crop legumes, such as soybean. Although, it is now universally accepted that, changes in endogenous and exogenous hormonal compositions cause dramatic effects on the entire regeneration process (Phat et al., 2015; Teale et al., 2006). Findings made in this study regarding the influence of seed vigour also complement the advances made in *in vitro* shoot induction and regeneration in soybean. All the studies have demonstrated that both biochemical and physiological processes within the seeds should be efficiently reinitiated and redirected in order to achieve the critical changes required for crop improvement via *in vitro* regeneration of plants.

3.3. In vitro elongation, rooting and acclimatisation

The correlation of seed vigour with elongation, rooting and acclimatisation of induced adventitious shoots has also been analysed. According to the results, there was no evidence of positive correlation or any direct relationship found between the response of subcultured shoots and seed vigour (Figure 5 e and f). Several researchers recommended the use of plant growth regulators for efficient responses during *in vitro* rooting and elongation (Olhoft and Somers, 2001; Zia et al., 2010; Raza et al., 2017). However, in this study, both shoot elongation and rooting were achieved on MS basal culture medium without hormones (Figure 5 e). Shoot elongation was observed on shoots and shoot clumps induced in all soybean cultivars regardless of shoots being regenerated from freshly harvested seeds or after 9-months of seed storage. Similar observations were also made on elongated shoots subcultured for rooting. Differences, however, were observed in both the *in vitro* elongation and rooting frequencies between individually subcultured shoots and shoots grown in clumps. All induced shoots that were cultured on elongation medium generally required a prolonged period of incubation in tissue culture. At the same time, individually excised adventitious shoots required shorter duration of incubation and showed rapid elongation with concomitant rooting in all cultivars regardless of the seed storage duration used (Figure 5 e).

In terms of root formation, variations were observed in the number of adventitious roots formed per shoot. Often, elongated shoots of cultivar LS677, LS678 and Peking were more vigorous in forming the roots than other soybean cultivars used. The successful rooting of *in vitro* derived shoots has been reported in most legumes. Eapen and George (1993) and Dayal et al. (2003) also reported root formation from shoots developed using various leaf and half-seed explants of pigeonpea (*Cajanus cajan* (L.) Millsp). Although, their observations did not provide any contrasting evidence with regard to the relationship between seed vigour and rooting, which is in line with the findings reported in this study. High proliferation capacity and vigorous development of roots remain highly beneficial for successful plantlet hardening and acclimatisation under *ex vitro* conditions (Figure 5 f). Furthermore, the efficiency of plantlet hardening, and acclimatisation could be attributed to the steps followed during this regeneration stage as reported by Texeira et al. (2011).

4. Conclusions

Many researchers do not consider seed vigour as a major obstacle, especially if the conditions of seed germination are optimum and meet all the germination requirements, such as under plant tissue culture. This study found that poor seed vigour had negative effects on the viability and responsiveness of explants for efficient shoot regeneration. As clearly indicated by the findings made in this study, seed vigour was found to be directly proportional to *in vitro* germination and induction of multiple shoots in soybean.

Declarations

Author contribution statement

Phetole Mangena: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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