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The Influence of Seed Viability on the Germination and In Vitro Multiple Shoot Regeneration of Soybean (*Glycine max* L.)

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Abstract: The moisture status of seeds is usually high during the period of harvest and deterioration (loss of viability) starts to occur when seeds are stored for longer periods. In the present study, soybean seeds were evaluated using a standard germination test, in vitro germination, and for efficient multiple shoot induction, following storage under ambient conditions for 0, 3, 6 and 9 months. Results showed that seeds stored for more than 3 months had reduced moisture content and decreased germination percentages in LS677, LS678, Dundee, Peking, TGx1740-2F and TGx1835-10E of the tested genotypes. In particular, seeds stored for 9 months showed significantly poor seed viability and less than 50% overall seed germination (Dundee—42%, LS678—49%, TGx 1740-2F—44%, TGx 1835-10E—48%), except for LS677 and Peking, with 52 and 55%, respectively. The efficiency of multiple shoot induction also decreased with prolonged seed storage, with all genotypes recording an overall decline from about 96% to 40% regeneration efficiency within 9 months. The results obtained clearly indicated that high germination rates and efficient in vitro shoot induction depended largely on seed viability and storage duration, and significantly differed according to genotypes.

Keywords: germination; seed storage; seed viability; seedling vigour; shoot multiplication; soybean

1. Introduction

Soybean (*Glycine max* L.) is one of the most important sources of high-quality plant-based proteins and oils used worldwide for manufacturing of health and consumable products. The high proportion of proteins (40%), lipids (20%) and water-soluble carbohydrates (10%) found in the seeds play a crucial role in human and animal welfare. In recent years, much attention has been paid to the improvement of growth and yield of soybean. The development of powerful tools, such as in vitro plant tissue culture that is used in plant regeneration and genetic modification of crops and medicinal and ornamental plant species, have been tested. Techniques such as in vitro shoot regeneration using cotyledonary node explants for multiplication and the production of new transgenic lines have provided useful insights for successful plant breeding programs. Such protocols have been applied in the attempt to improve soybean growth under biotic and abiotic stress. Multiple shoot induction using immature embryos [1], cotyledonary node explants with or without axillary buds [2], shoot organogenesis on mature and immature cotyledons [3] and the use of double cotyledonary node explants prepared from 10-day-old seedlings [4] have been established for in vitro regeneration. However, due to the myriad of constraining factors involving both tissue culture conditions and gene manipulation, these advances still require optimisation. The moisture status of the seeds is usually high during the period of harvest and then starts to decrease, especially when seeds are stored for longer periods, causing seed deterioration, also known as the loss of viability. Seed deterioration is one of the most important

constrains in the germination, seedling development and regeneration of soybean plants. The rate at which seed deterioration occurs and how seeds generally respond under in vitro plant tissue culture conditions depend on several factors, which include seed viability, age, storage conditions, storage period and the genotype. This phenomenon is one of the major factors that is neglected, and it is a continuous problem for many researchers dealing with soybean in vitro regeneration and transformation. Furthermore, the loss of seed viability is also proven to have negative impacts on subsistence and commercial farming, severely affecting field emergence of seedlings during soybean cultivation [5], subsequently decreasing growth and productivity of the crop. In plant tissue culture, the effect of seed viability is not fully researched, especially its role in influencing seed germination, normal seedling development and vigour of seedlings from which cotyledonary explants are derived. Many authors, including Keneda et al. [6], Paz et al. [7], Shelar et al. [8], Hartmann et al. [9] and Raza et al. [10] have highlighted the difficulties faced during in vitro regeneration of soybean, especially with regard to genotype specificity. These reports, as well as many others, paid less attention to the loss of seed viability, which occurs not only in soybean but even in *Galenia pubescens*, *Swertia chirayita* and *Zea mays* [11–13].

Therefore, the objective of this research was to determine the effects that seed viability has on germination, seedling vigour and multiple shoot induction. The potential of selected soybean genotypes for successful in vitro plant regeneration was also evaluated.

2. Materials and Methods

2.1. Plant Material

Soybean (*Glycine max* L.) seeds of the genotype Dundee, TGx1740-2F and TGx1835-10E were sourced from the Department of Plant Production, Soil Science and Agricultural Engineering at the University of Limpopo. Seeds of soybean LS677 and LS678 were purchased from Link Seed South Africa, and Peking seeds were obtained from the Department of Biodiversity, University of Limpopo. The acquired seeds were planted at the Amaloba nursery (Turfloup campus—University of Limpopo) to produce fresh harvests as seed source for use in standard germination test and in vitro plant tissue culture. Good quality seeds with no signs of disease infections or physical damage were selected, weighed, placed in paper bags and stored under ambient conditions at 24 ± 2 °C with $\leq 60\%$ humidity. The study was conducted in a specialised laboratory for Experimental Plant Tissue Culture in the Department of Biodiversity (Botany), at the University of Limpopo, from 2015 to 2017.

2.2. Effect of Seed Storage on Moisture and Standard Germination Test

Seed moisture content was analysed by comparing the fresh seed weight with the weight of the seeds after every three months of storage, before standard germination test and in vitro plant regeneration were conducted. Moisture content was expressed as the mean percentage moisture for each 100 seed lot. The equation below was used to calculate the seed moisture content:

$$\text{Seed moisture percentage} = \frac{\text{initial 100 seed mass}}{\text{mass of seeds after storage}} \times 100\%. \quad (1)$$

Prior to in vitro seed germination and plant regeneration, the harvested seeds were evaluated for viability using a standard germination method prescribed by the International Seed Testing Association [14] with modifications. Soybean seeds were laid on sterile damp filter papers in pre-sterilised glass petri dishes (150 mm) and covered with a single filter paper moistened with sterile distilled water. The seeds were incubated in a growth room for seven days at 24 ± 2 °C, $50\text{--}60 \mu\text{mol m}^{-2}\text{s}^{-1}$ light and 16 h photoperiod. Seed germination was recorded daily as the cracking of the seed coat and radicle emergence. A total of 200 seeds of each genotype were randomly separated into five sets of replicates, and the experiment was repeated three times. Seed viability analyses were carried out immediately after harvest (at 0 months) followed by 3, 6 and 9 months of seed

storage under ambient conditions. The germination and viability percentage were calculated using the following formula:

$$\text{Percentage germination} = \frac{\text{total no. of germinated seeds}}{\text{total no. of seeds tested}} \times 100\%. \quad (2)$$

The morphology of developed seedlings was visually assessed by recording the number of abnormal seedlings, evaluated as those that exhibited damage on essential structures such as the roots, epicotyls, hypocotyls and cotyledons. The percentage seed viability index and seedling vigour index (determined from the total number of normal seedlings) were calculated using the formulae:

$$\text{Seedling vigour index} = \frac{\text{mean radicle length} + \text{mean shoot length}}{\text{final percentage germination}} \quad (3)$$

$$\text{Percentage viability} = \frac{\text{total no. of viable seeds}}{\text{total no. of seeds tested}} \times 100\% \quad (4)$$

2.3. Effect of Seed Storage on In Vitro Germination and Seedling Development for Shoot Induction

Soybean seeds were surface-sterilised using chlorine gas according to Paz et al. [15]. Petri dishes (100 mm × 15 mm) containing seeds were placed in a desiccator jar in a fume hood. A 250 mL beaker containing 100 mL domestic bleach was placed into the jar with the seeds. An amount of 3.5 mL concentrated hydrochloric acid was carefully added into the beaker with bleach. The jar was immediately closed tightly, and the seeds were surface-sterilised with the liberated chlorine gas for 16 h. Sterilised seeds were inoculated on Murashige and Skoog (MS) basal culture medium for germination and seedling development. The MS culture medium was prepared as described by Pierik [16], modified with 2.00 mg L⁻¹ 6-benzylaminopurine (6-BA) as reported by Mangena et al. [4]. Other seeds were inoculated on MS basal culture medium without 6-BA, as a control. Each culture vessel contained three inoculated seeds and 30 replicates were made for each cultivar. Seed cultures were incubated in a growth room at 24 ± 2 °C and 50–60 μmol m⁻²s⁻¹ light intensity with 16 h photoperiod for 10 days. The experiment was repeated three times for 0, 3, 6 and 9 months of seed storage. Seed germination was recorded as the protrusion of the radicle, splitting of cotyledons and outgrowth of the epicotyls, and calculated using the equation below:

$$\text{Percentage germination} = \frac{\text{total no. of germinated seeds}}{\text{total no. of seeds tested}} \times 100\%. \quad (5)$$

2.4. Explant Preparation and In Vitro Shoot Induction

The 10-day-old seedlings were transversely cut on the hypocotyls, and their epicotyls excised off from the base at the cotyledonary junctions to obtain the double cotyledonary node explants [4]. The double cotyledonary node explants were subcultured for three weeks on MS medium containing 2.00 mg L⁻¹ 6-BA for the formation of multiple shoots. Stunted shoot clumps and buds were re-cultured on a fresh MS medium for more than 3 weeks until vigorous shoot growth was achieved.

2.5. Shoot Elongation and Rooting

After shoot formation, the induced multiple adventitious shoots were excised off the double coty-nodes and subcultured on full-strength hormone free MS medium for elongation. The elongated shoots were then transferred onto MS basal medium supplemented with 2.7 mg L⁻¹ indole-3-butyric acid (IBA) and 2.3 mg L⁻¹ α-naphthalene acetic acid (NAA) for adventitious root formation.

2.6. Ex Vitro Plant Establishment

The in vitro rooted plantlets were removed from culture medium, rinsed with sterile distilled water to wash off agar medium, and then transplanted to vessels containing sterile vermiculite. The plantlets were covered with transparent plastics, which was gradually removed to facilitate acclimatisation under controlled environmental conditions. The acclimatised plants were transferred into 25 cm plastic pots containing a mixture of fine sand (30%) and vermiculite (70%). All plants were maintained and grown at 24 ± 2 °C temperature, $150\text{--}200 \mu\text{mol m}^{-2}\text{s}^{-1}$ white light and 8 h dark to 16 h light period for four weeks. After this period, the plants with at least two trifoliolate leaves were transferred and kept in a glasshouse under natural conditions for hardening and further growth.

2.7. Experimental Design and Statistical Analysis

All experiments were completely randomised and repeated at least thrice with 30 replicates per treatment and genotype. The study was conducted using freshly harvested seeds (at 0 months) and the seeds stored for 3, 6 and 9 months under ambient conditions. All growth conditions were kept the same throughout the experiments, with plant tissue culture environment maintained, and natural conditions in the glasshouse were monitored but not controlled. Data collected on seed germination, seedling vigour, seed viability and shoot responses (number of shoots and roots) were recorded as growth parameters and evaluated after every three months of seed storage. The data was subjected to one-way analysis of variance (ANOVA) and their means compared using Duncan's multiple range test using IBM SPSS Statistics 24 (IBM South Africa, Grayston Sandton, South Africa).

3. Results

3.1. Effect of Seed Moisture Loss on Standard Germination Test

The results on seed germination during the standard germination test demonstrated a decline based on seed storage duration. The differences in germination percentage and seedling development resulted from the decrease in seed moisture content, as illustrated by the decrease in seed mass. There was a clear drop in seed mass as storage progressed, affecting percentage moisture content of the seeds as indicated in Table 1 and Figure 1. The results showed that high seed germination was observed mostly in freshly harvested seeds (0 months), and then decreased as a result of prolonged seed storage (Figure 1A–D). According to the standard germination results, soybean LS677 and LS678 genotypes consistently exhibited the highest germination percentage followed by Peking, Dundee, TGx1835-10E and TGx1740-2F. The TGx genotypes were the least performing genotypes amongst the soybeans tested, showing almost 25% drop in germination after three months of seed storage (76%—TGx 1835-10E and 78%—TGx 1740-2F) and then further decreased by 44% and 40%, respectively, at 9 months. Amongst the genotypes, Peking demonstrated a substantial loss in seed mass as shown in Table 1, subsequently causing a significant delay in germination. Nevertheless, this did not affect seedling development, as the percentage of normal seedlings remained above 60% for most of the experiment.

All soybeans presented some abnormalities in the shoots and roots of seedlings following prolonged seed storage. Cracking of cotyledons, undeveloped roots and highly reduced hypocotyls and epicotyls were some of the abnormal features (Figure 2C). These variations were more pronounced in seeds stored for 6 and 9 months, causing a significant drop in normal seedling percentage in all genotypes. This decline in seedling vigour appeared directly linked to a decline in seed viability and seed moisture content, as indicated on Table 1 and Figure 2. Furthermore, the loss in vigour resulted in seedling abnormalities, predominantly, in soybean Dundee, TGx1740-2F, TGx1835-10E and LS678.

Table 1. Change in seed mass measured from 100-seed lots of soybeans used for standard germination test over different durations of storage.

Cultivars	Seed Mass (g)				Seed Moisture (%)
	0 Months	3 Months	6 Months	9 Months	
Dundee	21.603 ^b	20.404 ^a	13.072 ^c	11.709 ^b	22.7 ^d
LS 677	24.842 ^a	18.099 ^c	16.493 ^a	10.741 ^d	29.4 ^b
LS 678	24.524 ^a	19.439 ^b	15.252 ^b	10.638 ^d	27.9 ^a
Peking	18.613 ^d	16.643 ^e	12.744 ^d	11.149 ^c	20.5 ^e
TGx 1835-10E	20.522 ^c	18.523 ^d	16.255 ^a	15.854 ^a	13.3 ^f
TGx 1740-2F	17.333 ^e	12.177 ^f	11.167 ^e	10.669 ^d	25.9 ^c

Mean masses followed by different letters are statistically different at 5% confidence interval according *t*-test. Seed moisture (%) was calculated from the mean mass of each seed lot using the Equation (1) in Section 2.2. Unit of measurement in brackets (g) refers to grams and (%) refers to percent.

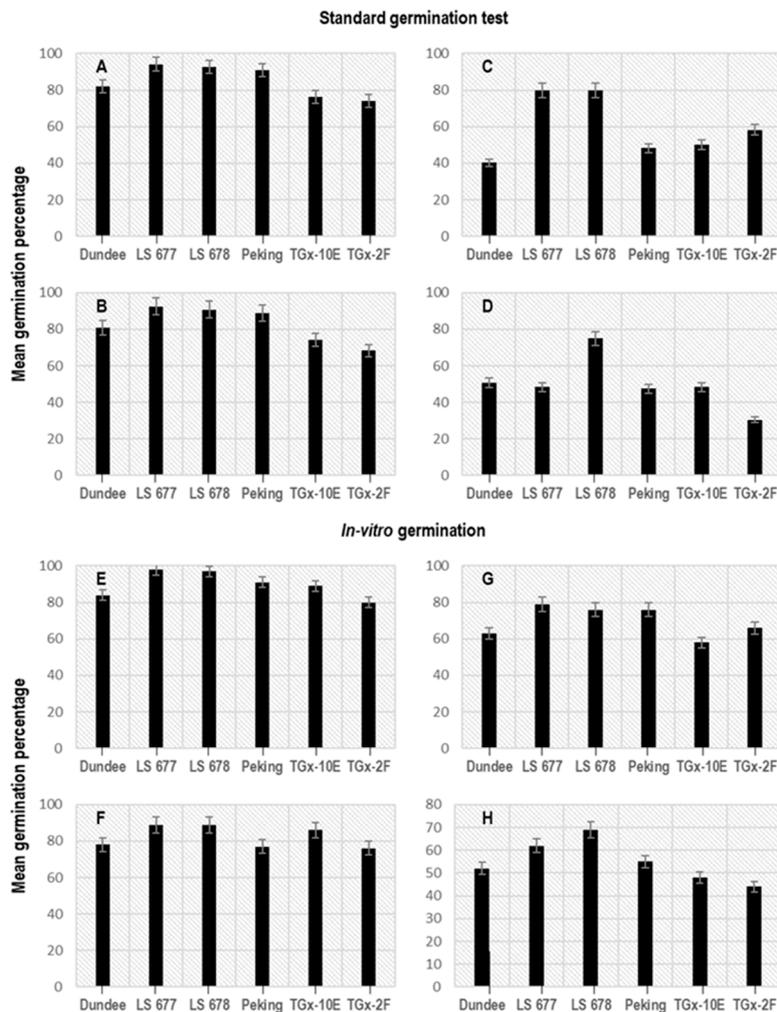


Figure 1. Mean germination percentage observed during the standard germination test and in vitro germination conducted after every three months of seed storage. (A–D) are percentage germination obtained at month 0, 3, 6 and 9 of seed storage under ambient conditions during standard germination test. (E–H) are germination percentages observed on MS culture medium supplemented with 2.00 mg/L 6-BA at 0, 3, 6 and 9 months of seed storage under ambient conditions.



Figure 2. Illustration of the sterilisation of seeds, standard germination test and in vitro regeneration stages followed in soybean cultivar Dundee, LS677, LS678, Peking, TGx1740-2F and TGx1835-10E. (A) Surface sterilisation of seeds. (B) Seedlings developed from 0 to 3 months of seed storage. (C) Example of poor germination at 6 to 9 months. (D,E) In vitro germinated seeds. (F,H) Multiple shoot induction on MS medium supplemented with 2.0 mg L^{-1} 6-BA. (G) Elongation of induced multiple shoots on MS basal medium containing IBA (2.70 mg L^{-1}) and NAA (2.30 mg L^{-1}). (I) Example of reduced shoot morphogenesis accompanied by callus induction on cotyledonary explants. (J) Example of full plant growth achieved during ex-vitro hardening and acclimatisation. (K) Mature fruit pods produced on in vitro regenerated plants.

3.2. Effect of Seed Storage on In Vitro Seed Germination

The seeds germinated on MS culture medium containing 2.00 mg L^{-1} 6-BA presented responses similar to the results observed during the standard germination test. Most of the germinated seeds in all soybean genotypes showed difficulties in the emergence of epicotyls, although their radicles were rapidly produced. At 0 to 3 months, seedling vigour was high as indicated (Table 2), producing good seedling characteristics with thicker and elongated hypocotyls and epicotyls. However, the presence of 6-BA in the medium resulted in the formation of reduced epicotyls, thicker hypocotyls and highly reduced primary roots without lateral roots. In general, poor seed germination and abnormal seedling development were predominant in experiments conducted with seeds stored for 6 and 9 months, as indicated in Figure 1E–H. The results clearly indicated that loss of seed moisture determined by the rapid loss of seed mass has severely affected seed viability and germinability, even from the onset of the experiment. All soybean genotypes indicated a loss in seed viability on MS culture medium as storage progressed, as shown in Figures 1 and 3.

Table 2. Effect of storage period on in vitro seedling establishment and multiple shoot induction. Seed germination and seedlings were developed on MS basal medium supplemented with 2.0 mg/L 6-BA and shoot induction achieved on MS medium containing 2.0 mg/L 6-BA.

Soybean Genotypes	0 Months			3 Months			6 Months			9 Months		
	SVI	MSN \pm SD	RE (%)	SVI	MSN \pm SD	RE (%)	SVI	MSN \pm SD	RE (%)	SVI	MSN \pm SD	RE (%)
Dundee	9.6 ^d	10.9 \pm 1.41 ^e	90.2 ^e	9.3 ^c	9.0 \pm 3.14 ^c	89.2 ^c	9.3 ^b	8.9 \pm 3.49 ^b	79.4 ^c	6.7 ^b	7.4 \pm 4.63 ^b	52.5 ^d
LS 677	12.5 ^a	13.5 \pm 0.35 ^a	96.5 ^a	9.8 ^a	13.0 \pm 1.56 ^b	96.0 ^a	9.6 ^a	9.4 \pm 3.43 ^a	83.6 ^b	8.9 ^a	8.0 \pm 4.79 ^a	70.9 ^b
LS 678	12.7 ^a	11.0 \pm 0.71 ^c	94.5 ^c	9.8 ^b	10.7 \pm 3.04 ^c	92.3 ^b	8.9 ^c	8.4 \pm 3.06 ^b	86.3 ^a	6.2 ^b	6.8 \pm 4.34 ^c	64.0 ^a
Peking	11.3 ^b	13.7 \pm 1.41 ^b	95.8 ^b	9.1 ^f	12.0 \pm 3.33 ^a	95.7 ^a	7.6 ^e	9.1 \pm 3.51 ^a	78.6 ^c	7.7 ^c	7.8 \pm 4.63 ^a	59.5 ^c
TGx 1835-10E	10.3 ^c	10.3 \pm 3.04 ^d	92.8 ^d	8.9 ^d	8.5 \pm 0.34 ^c	80.6 ^e	8.6 ^c	7.6 \pm 2.52 ^c	63.2 ^e	5.8 ^c	5.5 \pm 3.86 ^d	42.8 ^e
TGx 1740-2F	8.8 ^e	8.5 \pm 0.35 ^f	90.9 ^e	7.6 ^e	5.9 \pm 2.34 ^d	88.5 ^d	8.0 ^d	2.2 \pm 1.83 ^d	72.1 ^d	6.6 ^d	5.3 \pm 3.22 ^d	40.4 ^e

Means within columns with same letters are not significantly different at the 0.05 level of probability using *t*-test. SVI refers to seedling vigour index, MSN is the mean shoot number, SD is the standard deviation and RE is the percentage regeneration efficiency calculated as % of the mean number of explants inducing more than 5 shoots per explant.

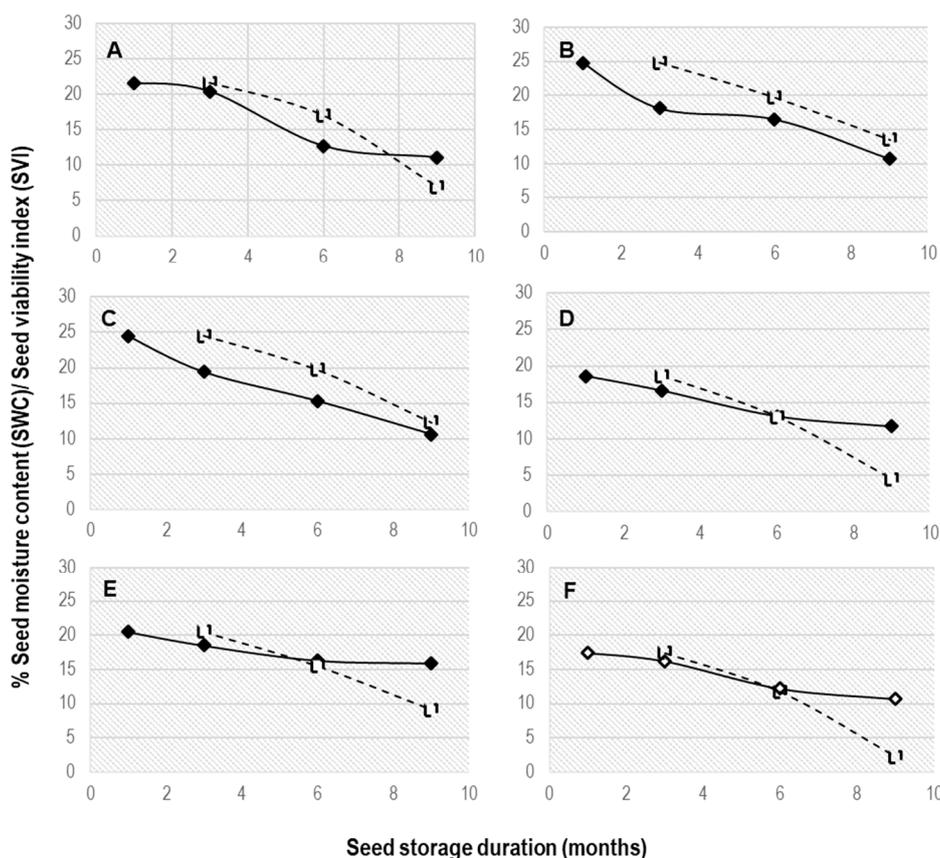


Figure 3. Seed moisture content (SMC) and viability (SVI) relations of the soybean seeds (A—Dundee, B—LS677, C—LS678, D—Peking, E—TGx 1835-10E and F—TGx 1740-2F) stored under ambient conditions. Solid line is the results of percentage moisture content of the seed during different storage periods and dashed line is the results of viability indices determined using the formulae as indicated in Section 2.2.

The effect of seed viability on *in vitro* soybean seed germination was significant and the highest seedling establishment index (SEI) were obtained in all genotypes during a short period of time (0 to 3 months). The results also indicated that percentage seed germination and SEI were affected by the genotype (Table 2). Although, the addition of 2.00 mg L^{-1} 6-BA on the germination medium had a profound effect on seedling growth, it did not influence the viability of the seeds and germination rate. On overall, germination percentage reached 80% and above, for all genotypes except for TGx1740-2F (76%) using freshly harvested seeds (0 months). A slight decline was then recorded for LS677, Dundee and TGx1835-10E after 3 months of storage, and then further declined for all soybeans after 6 and 9 months. Furthermore, the mean germination percentage and seedling establishment index were generally higher in LS677, LS678 and Peking than in Dundee, TGx1740-2F and TGx1835-10E, as indicated in Figure 1 and Table 2.

3.3. Effect of Seed Storage Duration on *In Vitro* Shoot Induction

Plant growth regulators (cytokinin) have been successfully used in this study to initiate cell division and differentiation on the double cotyledonary node explants. Multiple shoots were efficiently proliferated on MS culture medium supplemented with 2.0 mg L^{-1} 6-BA at 0 to 3 months (Table 2). The highest number of shoots induced per explant was achieved during the first experiment (at month 0 after harvesting) followed by shoot induction achieved using seeds stored for 3, 6 and 9 months (Table 2). The frequency of shoot formation was significantly influenced by the prolonged storage of the seeds, used as indicated on the correlations in Figure 3.

The MS culture medium composition and genotypes appeared to have caused considerable combined effects on shoot multiplication, including seed viability and seedling vigour, as indicated in Table 2 and Figure 3. However, the highest number of shoots with a mean value of 13.7 shoots per explant was obtained in Peking, suggesting high seed viability as well as an influence by the genotype and culture conditions. This was slightly higher than the mean number of shoots induced in LS677 (13.0), Dundee (10.9) and LS678 (11.0). A small variation was observed among these genotypes during the second experiment at month 3, where the number of shoots decreased to an average of 9.0, 13.0, 10.7 and 12.0 for Dundee, LS677, LS678 and Peking, respectively (Table 2). The mean shoot number continued to show a decline for the successive periods of seed storage (6 and 9 months). Soybean cultivar TGx1740-2F recorded the lowest mean number of shoots ranging from 2.2 to 5.9 per explant, followed by TGx1835-10E with about 5.5 to 10 shoots per explant for the entire experiment (Table 2).

3.4. *In Vitro* Shoot Elongation and Rooting

Shoot elongation and rooting was observed from shoots and shoot clumps initiated in all soybean genotypes (Figure 2F,H,I). There were, however, notable differences in the elongation and rooting between individually subcultured shoots and shoots that developed as clumps. Shoot clumps generally required a prolonged period of incubation on the elongation medium. On the other hand, individually excised shoots required shorter periods of incubation and showed rapid elongation that occurred within 2 weeks. There was no direct relationship observed between shoot elongation/rooting in relation to seedling vigour or the viability of the seeds used to develop cotyledonary explants. Furthermore, there was no correlation found between these growth characteristics with the storage of seeds for a prolonged time under ambient conditions. In cultivars such as TGx1740-2F and TGx1835-10E, rooting was gradual, especially on MS medium containing 2.70 mg L^{-1} IBA and 2.30 mg L^{-1} NAA than MS culture medium without PGRs. The formation of a compact green callus was observed on the stem bases, which inhibited root initiation contributing to the delay in rooting. Those soybean shoots showed light green callus cells as observed at the coty-node explant junctions in Figure 2I. The addition of IBA in combination with NAA proved to be very critical for the formation of adventitious roots, resulting into faster root induction without callus formation in Dundee, Peking, LS677 and LS678. These genotypes appeared highly responsive to the selected medium composition and combinations of plant growth regulators.

3.5. *Acclimatisation of Regenerated Plantlets*

After *in vitro* rooting of shoots, the plantlets were acclimatised and established in soil (Figure 2J,K). As for shoot elongation and rooting, acclimatisation of rooted plantlets was not affected by seed viability, culture conditions or genotype. However, in the case of plantlets regenerated from explants showing low proliferation capacity and less vigorous growth of shoots (TGx 1740-2F and TGx 1835-10E cultivars), rooted shoots took longer to acclimatise than those showing active growth. All poorly *in vitro* rooted plantlets showed difficulties in acclimatisation as a result of poor root development and exhibited higher sensitivity to each transfer step. Although most of the plantlets from these selected genotypes showed successful initiation of adventitious roots and shoots, many of them required more than 4 weeks of acclimatisation, especially those incapable of rapidly overcoming acclimatisation-related stress.

3.6. *Flowering and Pod Production*

More than 50% of plants which were successfully regenerated for all genotypes could be induced for flowering with subsequent fruiting (Figure 2K). The success in flowering also did not depend on seed viability or the genotype. However, the plants that were successfully elongated and rooted on medium containing hormones showed rapid vegetative and reproductive growth, probably influenced by 6-BA habituation. Poor flowering and fruit setting were observed mostly in cultivar Dundee and TGx1835-10E compared to the other soybean genotypes used. In addition, the responses were more

related to plant establishment than genotype, as several replicates demonstrated better flowering and pod production.

4. Discussion

The results obtained in this study have indicated that seed storage for a prolonged period under ambient conditions causes a decrease in seed viability, which has negative effects on standard seed germination, in vitro seed germination, seedling development and the frequency of in vitro shoot regeneration. Our results showed that seed moisture content, seed viability and seedling vigour, as well as shoot multiplication, decreased as seed storage increased for more than 3 months. This was supported by Malik and Yoti [17], indicating that seed longevity normally decreases as their storage duration under ambient conditions progresses. Furthermore, rapid deterioration of soybean seeds could take place if the seeds are maintained under high temperature (>30 °C) and high humidity (>60%) during storage. This deterioration was also reported by Rosenberg and Rinne [18], who further suggested that seed storage affects seed moisture content, as also shown by our results, which showed a decrease in seed mass with an increase in seed storage duration. Rosenberg and Rinne's observations were similar to the results obtained in this study. According to our findings and results by Rosenberg and Rinne [18], moisture is a prerequisite for better germination and seedling development. However, percentage germination and seedling establishment index could decrease with the loss of seed moisture following prolonged storage, irrespective of the genotype. Seed moisture above 18% is critical to maintain in order to avoid field, shatter and market losses in soybean [14]. In our study, extreme loss of seed viability observed over 9 months had a profound impact on seed moisture, thus negatively impacting the germination of seeds and subsequent seedling development in all soybean genotypes. The loss in seed viability was similarly reported to be common in many crops, causing problems in the production and expansion of recalcitrant legumes such as cowpea and soybean, as supported by Dadlani et al. [19], Shaban [20] and Shelar et al. [8]. These authors attributed seed deterioration to poor seed respiration, heating and possible microbial infections.

In general, the differential responses observed in the germination of seeds via standard germination test or in vitro germination revealed significant dissimilarities that exist amongst the genotypes. Peking, LS677 and LS678 cultivar provided superior response over Dundee, TGx1835-10E and TGx1740-2F, consecutively. These findings also support observations made by Shelar et al. [8], who indicated that seed viability in soybean varieties is very momentary and that this differs according to the genotype and age of the seeds. The evidence of seed performance based on genotypes have been previously reported by Jepleting [21] and Pinthus and Kimel [22]. Jepleting [21] additionally indicated that soybean seeds deteriorate rapidly, with deterioration rates varying according to storage conditions and initial seed quality in addition to the genotype factor. Seed viability was found to be very low in TGx1835-10E and TGx1740-2F compared to other genotypes, and this negatively influenced the germination and shoot induction, as described by Shelar et al. [8], Gleekia-kerkula [23] and Rajjou et al. [24]. Reports including those of Olhoft and Somers [25], Paz et al. [15] and Mangena et al. [4] established that successful regeneration of soybean plants relies primarily on strong seedling establishment from which viable explants are derived. However, this study indicated that the production of high vigour seedling also relied upon good seed quality emanating from prolonged seed viability.

Shoot growth proved to be supported and directly linked to seed quality, age and genotype. Furthermore, the percentage regeneration efficiency showed evidence of decrement as the viability of seeds started to decline. These made seed viability an important factor to be considered during the optimisation of protocols used for efficient in vitro regeneration of soybean and other recalcitrant crops. Based on the overall results obtained in this study, the genotypes demonstrated the following: (i) a decrease in seedling establishment index as seed storage duration increased, (ii) a decrease in the number of shoots induced as seed storage progressed, and (iii) differences in germination and multiple shoot induction according to genotypes in relation to seed storage under ambient conditions.

These stated outcomes provide a clear account of the effects of seed viability and storage duration for subsequent *in vitro* regeneration, which is not yet accounted for in plant tissue culture. The relationship between seed viability and plant establishment has been reported by Edelstein et al. [26] and Wang et al. [27], but these reports primarily focussed on field experiments. The results obtained in this study suggest that soybean seeds must be dried to a required moisture level between 18% and 30%, which should be maintained until crushing or planting, and the required moisture level may differ according to genotype or storage duration. These results suggest that, seeds of better physiological quality will induce higher germination rates, lesser abnormal seedlings, improved multiple shoot induction and regeneration under *in vitro* plant tissue culture [28].

5. Conclusions

The *in vitro* and standard germination of seeds, as well as the efficiency of *in vitro* induction of multiple shoots in soybean, could decline to less than 50% due to prolonged seed storage for more than 3 months. These decreases may furthermore differ according to genotype and moisture content of the seeds.

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