Asian Journal of Agricultural and Horticultural Research

Volume 10, Issue 4, Page 22-39, 2023; Article no.AJAHR.98974
ISSN: 2581-4478

Using Oxygen Bioavailability and Somatic Embryogenesis Techniques for Regeneration of Old Seeds of Common Bean (*Phaseolus vulgaris* L.)

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJAHR/2023/v10i4241

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/98974

Original Research Article

Received: 21/02/2023
Accepted: 23/04/2023
Published: 04/05/2023

ABSTRACT

The deterioration of the viability of seeds of old varieties (heirloom varieties) by exposure to long storage periods is one of the main problems facing plant breeding programmes; Thus, oxygen bioavailability and somatic embryogenesis techniques can overcome this problem to some extent. The goals of this study were to: 1) define the optimal oxygen bioavailability concentration for increasing the germination rates of old and waterlogged seeds; 2) define the relationship between the concentration of bioavailable oxygen in the liquid ingested by the germinating seed and seed vigor; and 3) use somatic embryogenesis techniques as a method for recovering plant germplasm from old or improperly stored seeds for use in plant breeding programs. This study was conducted in the tissue culture laboratory of the Potato and Vegetatively Propagated Vegetables Department,

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Horticulture Research Institute, Agricultural Research Centre, Egypt. This research was conducted to regenerate perennial seeds (8 years) of common bean (*Phaseolus vulgaris* L.) variety Giza 6 (local variety) and compared with 1 year old seeds as control treatment of the same cultivar. This study established that prolonged hypoxia throughout a protracted period of storage can inhibit germination. Hypoxia during the impregnation and germination of bean seeds can be overcome by supplying the seeds with hydrogen peroxide (20 mM) throughout the germination process. Catalase enzyme (10.5 units/mL) is needed to convert hydrogen peroxide into oxygen. Also, to release oxygen from hydrogen peroxide, other catalytic agents, such as transition metal ions like Fe^{+2}, Cu^{+2}, and Mn^{+2}, can be used at the following concentrations: 0.001M, 0.001M, and 0.0001M, respectively. A protective layer of $CaSO_4$ (0.5 mM) was applied since aged seeds have very fragile biological membranes. The test of tetrazolium gave results for some seeds that it non-viable or some parts of the seed are damaged or defective; However, 2,4-D at a concentration of 5 mg/l + supplements (0.1 µM GA_3/L + 2 mg kintien/L + 1.0 mM glutamine) was able to produce somatic embryos of the old seeds tissues for common bean cv. Giza 6 (a local variety). The study of the morphology and induction of somatic embryogenesis was done used scanning electron microscopy. In its early phases, the callus appeared as a scattering of elongated cells. And after being transferred to the DCR medium, the cells became tightly packed together, and globular-shaped embryos began to emerge between the tightly packed together cells.

Keywords: Common bean; Phaseolus vulgaris L.; aged seeds; somatic embryogenesis; regeneration old seeds; electron microscopy; tetrazolium test; 2,4-D.

1. INTRODUCTION

Phaseolus vulgaris L., or common bean, provides about 85% of all bean harvest worldwide. [1]. Almost 27 million tones-of common beans are produced annually on 29 million acres of land global [2], providing food for more than 300 million people connected to agricultural economies worldwide [3]. Damaged seeds lose their vigor and are more susceptible to stress during germination; eventually, the seeds lose their ability to germinate. Genetic makeup, physiological state, and storage conditions influence the rate of seed spoilage [4]. To build a successful seed production program and to maintain a thriving and productive agriculture, there must be a sufficient supply of high-quality seed. Unanticipated losses in seed viability would have a detrimental impact on storehouse stockpiles, production plans, and seed sales, causing agricultural enterprises to suffer enormous losses each year [5]. The rate at which seed health declines increases as seed moisture content and temperature rise noticeably. According to [6], seed storability is often impacted by seed quality at the time of storage, the seed's prior storage history, relative humidity, the length of storage time, the moisture content of the seed, storage temperature, and biotic variables. The two main factors that affect seed storability are seed moisture content and storage conditions [7]. The general definition of seed deterioration is "deteriorative changes happening over time that increase the seed's vulnerability to environmental stresses and reduce the seed's capacity to survive". Several physiological changes, such as impaired protein synthesis, lipid peroxidation, DNA damage, and membrane disruption are all part of the complicated process of seed degeneration. When the seed moisture content is hight, the deteriorative biochemical events happen quickly, posing a serious threat to the survival and longevity of seed offspring [8]. It is a series of occurrences characterized by several biochemical responses, mostly membrane damage and impairment of cellular machinery leading to a decrease in seed production process of germinability [5]. Seed degradation reduces the quality of the seed and damages its phenological and biochemical characteristics [9]. In turn, this causes seeds to become less viable, which could lead to the death of seedlings. When seed germinability declines, it causes a drop in the coefficient rate of germination. Many physiological, metabolic, and chemical changes, such as lipid peroxidation caused by free radicals, a decrease in protein content, the inactivation of protein biosynthesis enzymes, the disintegration of the cell wall, and RNA damage, all contribute to the process of seed deterioration [10]. Denaturalization of DNA leads to lipid peroxidation, which hinders protein transcription and translation and results in the oxidation of several amino acids. The rate of seed germination is slowed down when these kinds of injuries to the seed testa occur. The membrane is the primary site for the process of lipid

peroxidation because it is a lipid bilayer in nature. Reactive oxygen species (ROS), lipid peroxidation, and free radicals [11] scavenging enzymes are frequently cited as the primary causes of seed degradation. Also, they alter the proportion of unsaturated fatty acids, which has an impact on the lipid bilayer structure of cell membranes and raises membrane permeability by deactivating membrane-bound proteins. During the progression of seed deterioration, an increase in the total soluble sugar content of the leachate content was observed. Yet, the rise in storage period was the cause of in sugar leaching [12]. A significant loss of respirable substrate from damaged seeds may be indicated by extensive sugar leakage. ROS, which affect a variety of metabolic activities including respiration, CO_2 fixation, and gaseous exchange, are primarily responsible for degradation. Damage to seeds may be prevented by the widespread production of reactive oxygen species such as hydroxyl radicals (OH^-), superoxide radicals, and hydrogen peroxide (H_2O_2). These metabolic processes result in oxidative cell alterations [13] and increase the chances of mutation rate.

A sophisticated defense mechanism is present in the cells to guard against harm done as a result of seed degradation. Catalase, superoxide dismutase, peroxidase, and ascorbate peroxidase are antioxidant enzymes that catalyze the regeneration and formation reactions to scavenge the reactive oxygen species. The defensive mechanism is made up of several nonenzymatic and enzymatic mechanisms. According to [14], there is a stronger correlation between onion seed viability loss and decreased catalase activity. As a result, the decline in seed viability is connected with the decline in catalase activity, which also highlights the seed's low affinity for H_2O_2 [15]. These enzyme activity changes are crucial biomarkers for tracking the biochemical alterations brought on by deteriorative processes [16]. Biochemical methods have been used also to evaluate the quality of seeds to monitor the activity of the enzymes responsible for cell respiration and reserve mobilization. The respiratory capacity of the seed decreases due to a fall in an enzyme's activity, which affects the provision of energy and nutrients to the germination seeds. Seeds that degrade quickly exhibit damage to the membrane, proteins, and nucleic acids. Unsaturated fatty acid peroxidation is one of the main causes of the decline in seed viability and longevity. The primary cause of rapid seed deterioration is thought to be the auto-oxidation of lipids and the increase in the concentration of free fatty acids during storage time. When fatty acid hydrocarbon chains spontaneously oxidize in the presence of oxygen during autoxidation, reactive free radical intermediates known as hydroperoxides are produced [17-18]. The degree of damage analysis may be used to forecast how well a seed lot will germinate. It is well recognized that one of the main areas of decline during seed aging occurs in mitochondria. Initiation of anaerobic respiration is anticipated to provide the energy for metabolic functions, if not for repair when mitochondria become dysfunctional. Moreover, multiple researchers found evidence of a connection between ethanol production and seed damage [19].

The goals of this study were to 1) define the optimal oxygen bioavailability for increasing the germination rates of old common bean seeds; 2) define the relationship between the concentration of bioavailable oxygen in the liquid ingested by the germinating seed and seed vigor; and 3) use somatic embryogenesis techniques as a method for recovering plant germplasm from old or improperly stored seeds for use in plant breeding programs.

2. MATERIALS AND METHODS

This study was conducted in the tissue culture laboratory of the Potato and Vegetatively Propagated Vegetables Department, Horticulture Research Institute, Agricultural Research Centre, Egypt, In the period from 2017 – 2019. This study was conducted to regenerate old common bean seeds (*Phaseolus vulgaris* L.) (8 years old) Compared to one-year-old seeds of the same variety Giza 6 (local variety). Several preliminary experiments were conducted to overcome some technical problems such as contamination, and deformed embryos, and to choose the appropriate protocol to stimulate seed germination. Seeds were obtained from the Vegetable Seed Production Unit, Vegetable seed production technology research department, Dokki, Giza, Egypt.

To differentiate between viable seeds and old seeds, there is a test called tetrazolium test (TZ) based on differentiation by color (red) so that the highly viable seeds are completely stained in red, then the degree of staining varies according to seed viability.

2.1 Tetrazolium Test (TZ)

2.1.1 Viable seeds

1. Completely stained seeds.
2. Seeds where the unstained, flaccid, and/or necrotic tissue does not exceed the permitted maximum area.

2.1.2 Non-Viable seeds: (so-called large damages)

1. seeds where the unstained, flaccid, and/or necrotic tissue does exceed the permitted maximum area
2. seeds with damage at any other essential area (eg. connecting area of cotyledons)
3. completely unstained seeds
4. empty seeds
5. rotten seeds
6. seeds infected with insects
7. seeds with deformed embryos

2.2 Method

Terazolium tests are performed on a sample of 200 seeds. They were punctured and then soaked in a 1% tetrazolium solution for 16 hours (Picture 1).

2.2.1 Proposed protocol to improve vigor and germination of aged common bean seeds

The protocol includes four main steps:

1. The first step was carried out using the seeds as they came from the field. To eliminate external pathogens, seeds were surface sterilized by 70% ethanol for 30 seconds, followed by 1% sodium hypochlorite for 10 min. One drop of Tween 80 was added to the Na-Hypochlorite solution to lower the surface tension. Seeds were then rinsed with sterile water three times. 30 seeds of uniform size were submersed in one liter of hydrogen peroxide solution (20 Mm) (in a 1.5 L beaker), then the beaker were kept in the dark at 25˚C in a growth chamber for 24 h of imbibitions. The hydrogen peroxide solution was supplemented with Catalase enzyme (10.5 unit/mL) for breaking down hydrogen peroxide to generate oxygen and various transition metal catalysts (Fe^{+2}, Cu^{+2}, Mn^{+2}). Metal catalysts were prepared using $FeSO4{\cdot}7H_2O_2$ (0.001M), $CuSO_4{\cdot}5H_2O$ (0.001M), $MnSO_4{\cdot}H_2O$ (0.0001M [21], and $CaSO_4$ (0.5 mM) was added to protect the membrane; whereas, aged seeds have very fragile biological membranes [22].
2. For improving vigor and softening of old seeds, the seeds obtained from the previous step were divided into two groups; group one: seeds were rinsed four times with sterile distilled water, allowing 5 min for each rinse. seeds were then inoculated in the Jars containing sterilized filter paper (Whatman No.1) or saturated cotton with Murashige and Skoog Basal Medium [23] + 0.1 µM GA_3/L + 2 mg kintien/L + 1.0 mM glutamine (M1 medium for germination). Five seeds per Jar were incubated at 25±1◦C under complete darkness until complete germination.
3. Group two (This group will be used to produce embryonic callus): Finally the sterilization steps were conducted to eliminate those pathogens exposed through the embryo dissection of embryo borne. Embryos were surface sterilized for 2 min with 15% commercial bleach (v/v) and rinsed 4 times with sterile water. This multiple-step sterilization method is reliable, reproducible, and efficient. For callus induction, mature embryos from imbibed and dehulled seeds were aseptically moved (not set free) slightly with a scalpel according to [24]. The seeds, together with their moved

viable seed | Non-viable seed | Non-viable seed | Root damage | Dead seed

Picture 1. A sample evaluation scheme for testing seed viability by the tetrazolium test
(source: [20])

embryos, were placed furrowed downwards. The sterilized explants were cultured on MS medium, containing 3% (w/v) sucrose and supplemented with 2,4-D (2,4-Dichlorophenoxyacetic acid) at a concentration of 2.5 or 5.0 mg/L + 0.1 µM GA_3/L + 2 mg kintien/L + 1.0 mM glutamine. The vessels used for the solid cultures were tubes with a capacity of 25 ml and jars with a capacity of 30 ml basal nutrient medium, respectively. The solid medium contained 0.8 % agar. The pH was adjusted to 5.7 ± 0.1 before autoclaving at 121°C for 15 min. Media incubated at 25± 1°C and constant illumination at 60 µmol/m2/s. This stage took about 6 weeks.

4. Once the embryos were formed and reached the cotyledonary stage, they were transferred to the germination DCR medium (Douglas–fir Cotyledon Revised medium) without growth regulators; the DCR medium contains macro- and micronutrients as described by [25]. This medium was developed to promote shoot proliferation. This formulation has ~1/4 the concentration of NH_4NO_3 and KNO_3 compared to MS. Additional Ca^{+2} and NO^{-3} ions are supplied by $Ca(NO_3)^2$. Embryos were picked with the help of sterile forceps and transferred to Petri dishes containing DCR medium. Once they formed plantlets each plantlet was transferred to the culture jar (PhytoTechnology) containing the MSO medium.

2.2.2 Studied traits

Tetrazolium test: Viable seed (n), Non-viable seed (n). Germination traits: germination percent (%), normal seedlings (n), abnormal seedlings (n), plumule length (cm), radicle length (cm), plumule fresh weight (g), radicle fresh weight (g), plumule dry weight (g), radicle dry weight (g). Somatic embryo stage: callus formation (%), embryo formation (%), Torpedo-shaped stage embryos (n/seed), plantlet numbers transferred to soil (n), survived plants numbers (n). These measurements were obtained as an average of the number per jar produced from one germinated seed. Three jars were taken at random, representing one replicate of each procedure.

2.2.3 Scanning electron microscopy

To prepare specimens for scanning electron microscopy, they were fixed for 4 hours at 4°C in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), dehydrated in ethanol using a critical point drier, and then coated with gold in an ion coater [26]. The specimen was examined under a scanning electron microscope, and photos of it were taken.

2.2.4 Counting the somatic embryo-stages

The embryonic callus developing on a solid medium was transferred to a suspension culture at the maturation of embryo tissue (ET), it was prepared as described in the materials and methods [27]. Briefly, the suspension cultures were initiated by transferring 1 g ET to 30 mL of liquid proliferation medium in 100 mL Erlenmeyer flasks that were subsequently placed on an orbital shaker at 90 rpm. After 7 days of subculturing, the suspension cultures (2 mL) were spread onto the top of a stack of five pieces of sterilized filter paper. After the liquid medium was absorbed, the filter paper contained the cultures. The evaluation was done by counting the torpedo-stage somatic embryos formed in each plot, with the aid of a stereo microscope. Torpedo-shaped embryos were defined as those with an axial length greater than 900 mm, without root formation. Cotyledonary-stage embryos were defined as those with root formation. Twin embryos (those with two cotyledonary parts), usually classified as abnormal embryos, were counted as normal in the present study because they were observed to grow into plants [28].

2.3 Experimental Design

This study was carried out in two experiments. The experiments followed a completely randomized design, with three replications per procedure. Experimental data were statistically analyzed using analysis of variance (ANOVA). **The first experiment**: This experiment was carried out on seeds germinated at the second step of the previously mentioned protocol. In this experiment, the statistical analysis consisted of a two-factor experiment (treatments and trials) with two treatments; the first treatment is germination improving and softening of old seeds treatment (H_2O_2 solution) not followed by the M1 medium for germination treatment, the second treatment is germination improving and softening of old seeds treatment (H_2O_2 solution) followed by the M1 medium treatment, compared with the control treatment (One-year-old seeds that were cultured in distilled water began to germinate). **The second experiment**: This experiment was carried out on seeds germinated after the second

step of the previously mentioned protocol. In this experiment, the statistical analysis consisted of a two-factor experiment (treatments and trials) with two treatments; the treatments are concentration of 2,4-D (2.5 and 5.0 mg/L of medium) + supplements compared with the control treatment (MS medium). The acquired data were statistically evaluated using Fisher's analysis of variance (given as a pairwise comparison procedure called the least significant difference (LSD) test). This test should be employed only if the overall F test rejects the hypothesis that all means are equal. If the overall test is significant, any pair of means is tested using a process similar to a standard Student's t-test. No additional tests are run if the total F ratio is not significant. When it is used, the two treatments are deemed different if the absolute difference between the two sample means is more than 5% using combined ANOVA across experiments with one-way complete randomized analysis (Multiple comparisons and trends among treatment means). The data from the two experiments were statistically analyzed using the CoStat package program, version 6.4 [29]. The differences among the means of treatments were tested using the least significant differences (LSD) at 0.05 level of probability according to the method described by [30,31]. The experiment was repeated twice (two trials) and the results of both experiments showed the same tendency.

3. RESULTS

The results obtained from the germination traits values resulting from the experiment of regeneration of old seeds germinated with distilled water only did not have any response in terms of improving the percentage of germination; also, the results obtained from the embryonic callus induction procedure (after 45 days) of cultivation on a hormone-free MS medium showed that the embryonic callus did not form, and therefore these procedures were not included in the statistical analysis process.

Mean squares and probabilities for combined ANOVA over two trials and the different procedures for tetrazolium test, regeneration of old seeds, and producing Somatic Embryogenesis in the common bean are presented in Table 1 and showing that all measured traits varied significantly among the procedures for regeneration of old seeds and producing Somatic Embryogenesis. There was no significant difference among the trials, except for the trait Callus formation and the trait number of plantlets transferred to soil both showed a significant difference. There were no significant differences among interactions of treatments X trials. The absence of significant differences among interactions of treatments X trials indicated that results were similar in both experiments.

R-squared can take any value between 0 to 1. The most common interpretation of r-squared is how well the regression model explains observed data. For example, an r-squared of 60% reveals that 60% of the variability observed in the target variable is explained by the regression model. Generally, a higher r-squared indicates more variability is explained by the model. The coefficient of variation shows the extent of variability of data in a sample to the mean of the population.

As shown in Table 2 and Picture 2, a statistically significant difference exists in the tetrazolium test for the fresh seeds and old seeds. Based on the comparison of the mean value, the statistically highest numbers of viable seeds (88.166) were counted in the fresh seed.

There was a significant variation in respect of germination percent (%), number of normal seedlings, number of abnormal seedlings, plumule length (cm), radicle length (cm), plumule fresh weight (g), radicle fresh weight (g), plumule dry weight (g), radicle dry weight (g) due to different treatments of distilled water, H_2O_2 solution, and H_2O_2 solution + M1 medium. The best results were achieved with the H_2O_2 solution + M1 medium treatment concerning all of the previous traits. The values were as follows; (20.16%, 19.16, 1.0, 6.3 (cm), 10.300 (cm), 0.990 (g), 0.306 (g), 0.084 (g), and 0.034 (g), respectively) (Table 3 and Pictures 3-8).

There was a significant variation in respect of callus formation (%), embryo formation (%), number of torpedo-shaped stage embryos per seed, number of plantlets transferred to soil, and number of survived plants due to different treatments of 2.50 mg/L (2,4-D) + supplements and 5 mg/L (2,4-D) + supplements. The best results were achieved with the 5 mg/L (2,4-D) + supplements treatment concerning all of the previous traits. The values were as follows; (21.466 %, 12.316 %, 152.67, 83.0, and 75.0, respectively) (Table 4 and Pictures 9-28).

The study of the morphology and induction of Somatic Embryogenesis was done used

scanning electron microscopy. In its early phases, the callus appeared as a scattering of elongated cells. And after being transferred to the DCR medium, the cells became tightly packed together, and globular-shaped embryos began to emerge between the tightly packed together cells.

Table 1. Mean squares and probabilities for combined ANOVA over two trails and the different treatments for tetrazolium test, germination traits, and producing somatic embryogenesis traits for old seeds of common bean

Traits	Sources of variation	df	MS	F	P
Number of viable seed	Main Effects				
	Treatments	1	21590.083	3454.413	***
	Trails	1	0.083	0.013	ns
	Interaction				
	Treatments * Trails	1	0.083	0.013	ns
	Error	8	6.25<-		
Germination percent (%)					
	Main Effects				
	Treatments	2	10453.722	2650.239	***
	Trails	1	0.889	0.225	ns
	Interaction				
	Treatments * Trails	2	1.388	0.352	ns
	Error	12	3.944<-		
Number of normal seedlings					
	Main Effects				
	Treatments	2	10602.056	2981.828	**
	Trails	1	0.5	0.140	ns
	Interaction				
	Treatments * Trails	2	1.166	0.328	ns
	Error	12	3.556<-		
Number of abnormal seedlings	Main Effects				
	Treatments	2	1.388	6.25	***
	Trails	1	0.055	0.25	ns
	Interaction				
	Treatments * Trails	2	0.056	0.25	ns
	Error	12	0.222<-		
Plumule length (cm)					
	Main Effects				
	Treatments	2	11.501	108.392	***
	Trails	1	0.08	0.753	ns
	Interaction				
	Treatments * Trails	2	0.021	0.204	ns
	Error	12	0.106<-		
Radicle length (cm)					
	Main Effects				
	Treatments	2	21.179	38.522	***
	Trails	1	0.393	0.715	ns
	Interaction				
	Treatments * Trails	2	0.054	0.098	ns
	Error	12	0.549<-		

,, ns = Significant ,highly Significant, and non significant at P< 0.05*

Cont. Table 1.

Traits	Sources of variation	df	MS	F	P
Plumule fresh weight (g)					
	Main Effects				
	Treatments	2	2.107	353.155	***
	Trails	1	0.001	0.244	ns
	Interaction				
	Treatments * Trails	2	0.005	0.974	ns
	Error	12	0.006<-		
Radicle fresh weight (cm)					
	Main Effects				
	Treatments	2	0.041	78.244	***
	Trails	1	0.005	9.672	ns
	Interaction				
	Treatments * Trails	2	0.001	2.201	ns
	Error	12	5.342e-4<-		
Plumule dry weight (g)					
	Main Effects				
	Treatments	2	0.001	266.202	***
	Trails	1	1.388e-6	0.337	ns
	Interaction				
	Treatments * Trails	2	9.055e-6	2.202	ns
	Error	12	4.111e-6		
Radicle dry weight (g)					
	Main Effects				
	Treatments	2	8.882e-4	499.625	***
	Trails	1	1.388e-6	0.781	ns
	Interaction				
	Treatments * Trails	2	5.555e-6	3.125	ns
	Error	12	1.777e-6<-		
Callus formation (%)					
	Main Effects				
	Treatments	**1**	21.600	52.050	***
	Trails	1	24.940	60.098	***
	Interaction				
	Treatments * Trails	1	0.700	1.688	ns
	Error	8	0.415<-		
Embryo formation (%)					
	Main Effects				
	Treatments	**1**	3.740	23.502	***
	Trails	1	8.333e-4	0.005	ns
	Interaction				
	Treatments * Trails	1	0.187	1.178	ns
	Error	8	0.159<-		
Number of torpedo-shaped stage embryos per seed	Main Effects				
	Treatments	**1**	616.333	85.011	***
	Trails	1	5.333	0.735	ns
	Interaction				
	Treatments * Trails	1	1.333	0.183	ns
	Error	8	7.25<-		

, , ns = Significant ,highly Significant, and non significant at P< 0.05*

Cont. Table 1.

	Sources of variation	df	MS	F	P
Number of plantlet transferred to soil	Main Effects				
	Treatments	1	408.333	222.727	***
	Trails	1	96.333	52.545	***
	Interaction				
	Treatments * Trails	1	8.333	4.545	ns
	Error	8	1.833<-		
Number of survived plants	Main Effects				
	Treatments	1	494.083	104.017	**
	Trails	1	0.75	0.157	ns
	Interaction				
	Treatments * Trails	1	44.083	9.280	ns
	Error	8	4.75<-		

, , ns = Significant ,highly Significant, and non significant at P< 0.05*

Table 2. Comparing the mean values for the tetrazolium test on fresh and old common bean seeds

	Treatments	Mean	Coefficient of variation (CV)	Coefficient of determination (R^2)
Number of viable seed	**Fresh seeds**	88.166[a]		
	Old seeds	3.333[b]	5.464%	0.997
	LSD 0.05	3.328		

Means within same column followed by the same letter are not significantly different at 5% by LSD

Table 3. Comparing the mean values of germination traits by using some treatments to regenerate old common bean seeds

	Treatments	Mean	Coefficient of Variation (CV)	Coefficient of determination (R^2)
Germination percent (%)	Fresh seeds + distilled water (control)	86.33[a]	5.151%	0.997
	Old seeds + H_2O_2 Solution	9.16[c]		
	Old seeds + H_2O_2 Solution then M1 medium	20.16[b]		
	LSD 0.05	**2.498**		
Number of normal seedlings	Fresh seeds + distilled water (control)	85.33[a]	5.058%	0.997
	Old seeds + H_2O_2 Solution	7.33[c]		
	Old seeds + H_2O_2 Solution then M1 medium	19.16[b]		
	LSD 0.05	**2.371**		
Number of abnormal seedlings	Fresh seeds + distilled water (control)	1.0[b]	36.89%	0.524
	Old seeds + H_2O_2 Solution	1.83[a]		
	Old seeds + H_2O_2 Solution then M1 medium	1.0[b]		
	LSD 0.05	**0.592**		
Plumule length (cm)	Fresh seeds + distilled water (control)	7.783[a]	5.116%	0.947
	Old seeds + H_2O_2 Solution	5.016[c]		
	Old seeds + H_2O_2 Solution then M1 medium	6.3[b]		
	LSD 0.05	**0.409**		
Radicle length (cm)	Fresh seeds + distilled water (control)	11.56[a]	7.481%	0.866
	Old seeds + H_2O_2 Solution	7.867[c]		
	Old seeds + H_2O_2 Solution then M1 medium	10.30[b]		

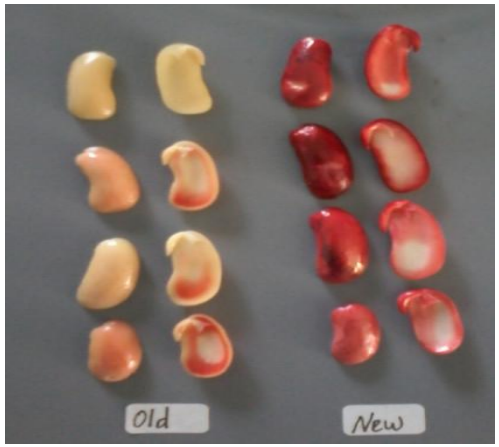
	Treatments	Mean	Coefficient of Variation (CV)	Coefficient of determination (R^2)
	LSD 0.05	**0.932**		
Plumule fresh weight (g)	Fresh seeds + distilled water (control)	1.890[a]	6.344%	0.983
	Old seeds + H_2O_2 Solution	0.772[c]		
	Old seeds + H_2O_2 Solution then M1 medium	0.990[b]		
	LSD 0.05	**0.097**		
Radicle fresh weight (g)	Fresh seeds + distilled water (control)	0.362[a]	7.993%	0.934
	Old seeds + H_2O_2 Solution	0.198[c]		
	Old seeds + H_2O_2 Solution then M1 medium	0.306[b]		
	LSD 0.05	**0.029**		
Plumule dry weight (g)	Fresh seeds + distilled water (control)	0.097[a]	2.421%	0.978
	Old seeds + H_2O_2 Solution	0.07[c]		
	Old seeds + H_2O_2 Solution then M1 medium	0.084[b]		
	LSD 0.05	**0.002**		
Radicle dry weight (g)	Fresh seeds + distilled water (control)	0.046[a]	3.877%	0.988
	Old seeds + H_2O_2 Solution	0.022[c]		
	Old seeds + H_2O_2 Solution then M1 medium	0.034[b]		
	LSD 0.05	**0.001**		

Means within same column followed by the same letter are not significantly different at 5% by LSD

Table 4. Comparing the mean values of producing Somatic Embryogenesis traits by using some treatments to induction of embryonic callus from old common bean seeds

	Treatments	Mean	Coefficient of Variation (CV)	Coefficient of determination (R^2)
Callus formation (%)	2.50 mg/L (2,4-D)+supplements	18.783[b]	3.201%	0.934
	5 mg/L (2,4-D) + supplements	21.466[a]		
	LSD 0.05	**0.857**		
Embryo formation (%)	2.50 mg/L (2,4-D)+supplements	10.433[b]	4.040%	0.755
	5 mg/L (2,4-D) + supplements	12.316[a]		
	LSD 0.05	**0.531**		
Number of torpedo-shaped stage embryos per seed	2.50 mg/L (2,4-D)+supplements	138.33[b]	1.850%	0.914
	5 mg/L (2,4-D) + supplements	152.67[a]		
	LSD 0.05	**3.584**		
Number of plantlet transferred to soil	2.50 mg/L (2,4-D)+supplements	71.33[b]	1.754%	0.972
	5 mg/L (2,4-D) + supplements	83.0[a]		
	LSD 0.05	**1.802**		
Number of Survived plants	2.50 mg/L (2,4-D)+supplements	62.16[b]	3.177%	0.934
	5 mg/L (2,4-D) + supplements	75.0[a]		
	LSD 0.05	**2.901**		

Means within same column followed by the same letter are not significantly different at 5% by LSD



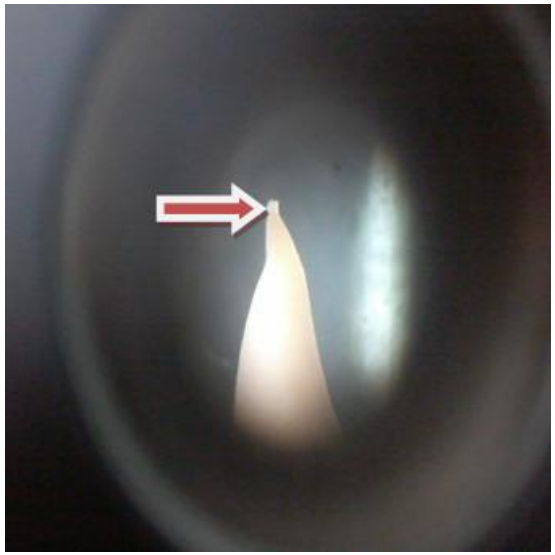
Picture 2. The Picture shows the difference between the degree of staining in the tetrazolimium test for both old and fresh seeds

Picture 3. The Picture shows seed imbibitions in 20 mM hydrogen peroxide solution and supplements after 24 h

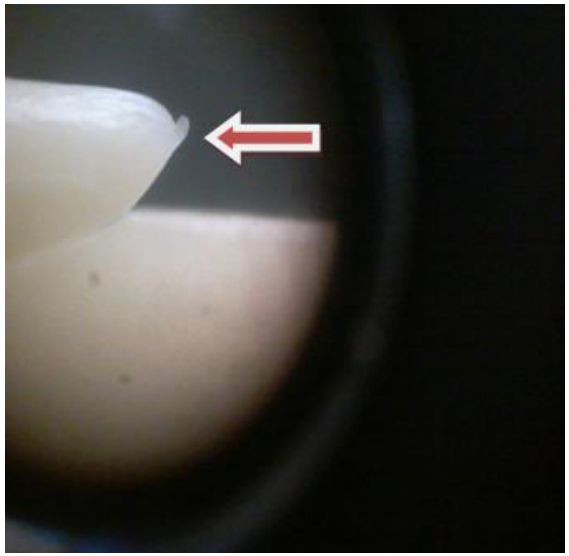
Picture 4. The Picture shows seed germinating and hypocotyls growth in 20 mM hydrogen peroxide solution and supplements

Picture 5. The Picture shows seed germinating and hypocotyls growth in 20 mM hydrogen peroxide solution and supplements

Picture 6. The Picture shows seed germinating and hypocotyls growth in 20 mM hydrogen peroxide solution and supplements



Picture 7. The Picture shows swelling of the root cap area of the embryo for the old seeds after imbibition



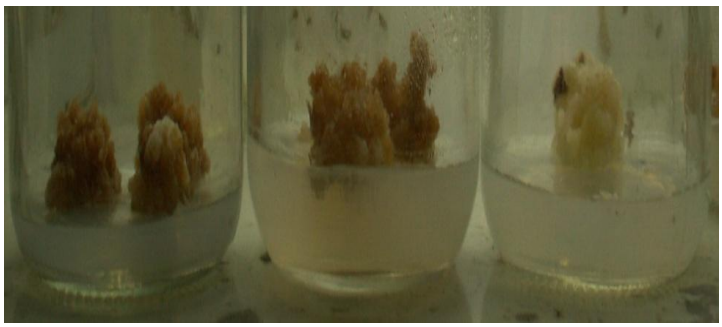
Picture 8. The Picture shows swelling of the root cap area of the embryo for the old seeds after imbibition

4. DISCUSSION

Some previous papers support and in agreement with our finding in this study. Before planting, seeds can be immersed in a solution for a few hours or days to introduce compounds that promote germination. It has been discovered that additional compounds can boost germination rates. When used with aged or big seeds, hydrogen peroxide can promote germination by increasing oxygen availability to developing embryos [32]. In addition to providing macronutrients, potassium nitrate may also assist in regulating plant hormones in the germination of seeds [33]. A hormone that occurs naturally in plants, gibberellic acid (GA), positively regulates germination and plant growth. GA is produced by a healthy seed as it absorbs water. In rare instances, GA treatments can speed up the emergence of germination indicators and boost germination rates in old or dormant seeds [34]. As an alternative, soaking seeds may lower the levels of Abscisic acid (ABA) in the seed coats, increasing GA activity [35]. Certain older seeds will sprout more readily if they are initially allowed to absorb moisture very slowly, preventing them from germinating, and then gently dried back to storage moisture content (that technique called seed priming). Osmopriming (soaking seeds in an osmotic solution) is a method that has been found to improve seed germination, even in older seeds, albeit the exact process is still unclear. Moreover, there are methods for bacterial seed priming that have been effective in boosting germination. [36] indicate that soaking medicinal plant seeds in a suspension of *Bacillus polymyxa* for twenty minutes before sowing improved the germination rate, germination speed, and early growth of growing plants. For several species, "bio-priming" with a bacterial suspension has been demonstrated to be effective. Although some microorganisms that encourage germination are now commercially accessible as a seed treatment that farmers can employ, priming requires laboratory equipment and expertise.

Starting seedlings on sterile agar growth media in Petri plates in indoor growth chambers can boost germination rates [37]. To provide the seeds with some of the nutrients and minerals that the seeds are unable to fully give, the medium can be supplemented with carbohydrates, amino acids, minerals, vitamins, and hormones [38]. For seed and tissue culture, a typical media is Murashige and Skoog (MS) [39]. The seeds will probably be cleaned or treated to decrease bacterial and fungal spores on the hulls, and the agar medium may contain antibiotics and/or fungicides to manage infections.

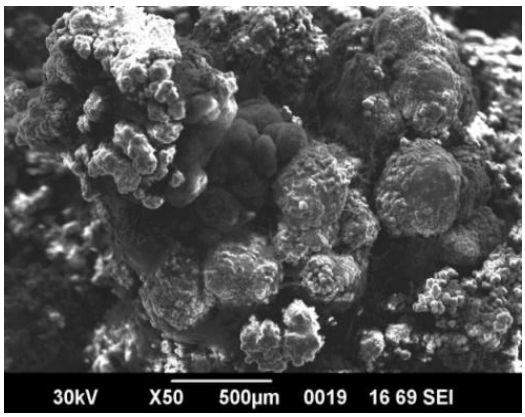
When somatic embryos (SE) are induced in plant culture systems, the addition of Plant Growth Regulators (PGR) to the culture media is crucial for promoting cell differentiation. The type and concentration of PGR employed for each culture affect the SE process most. Using various explants, circumstances, and PGR concentrations, various plant species, including *C. canephora* [40], *A. thaliana* [41], and *Musa spp.* [42], successfully responded to the SE induction. Auxins must be added to the culture medium for many species that may develop somatic embryos from cell suspension cultures. Inducing SE and the early stages of somatic embryo development requires the use of 2,4-dichloroacetic acid (2,4-D) [43]. For instance, by applying a low concentration of 2,4-D, the production for embryogenic date palm harvests improved 20 times [44]. Other PGRs, like cytokinins (CKs), also contribute to the growth of plants by encouraging the development of buds, delaying the aging of the leaves, and boosting cell division in conjunction with auxins; these two regulators are known to work in concert [45].



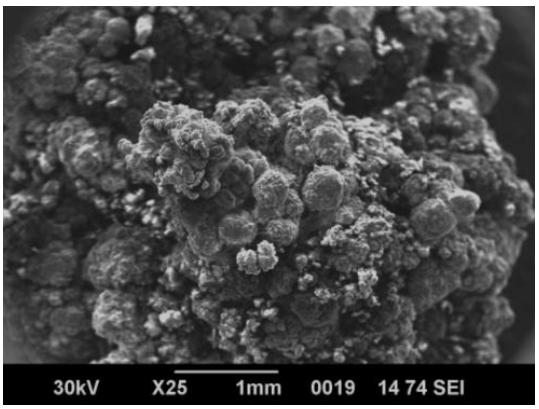
Picture 9. The Picture shows the stage of embryonic callus formation when the full parts of the seeds are incubated on 5 mg/L (2,4-D) + supplements medium

Picture 10. The Picture shows the stage of multiplication when the full parts of the seeds are incubated on 5 mg/L (2,4-D) + supplements medium

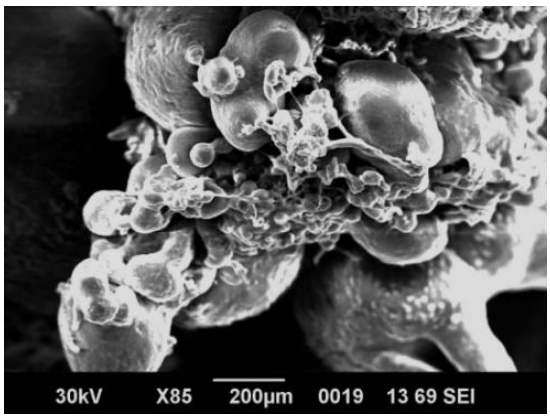
Picture 11. The Picture shows the stage of multiplication and hypocotyls growth when the full parts of the seeds are incubated on 5 mg/L (2,4-D) + supplements medium



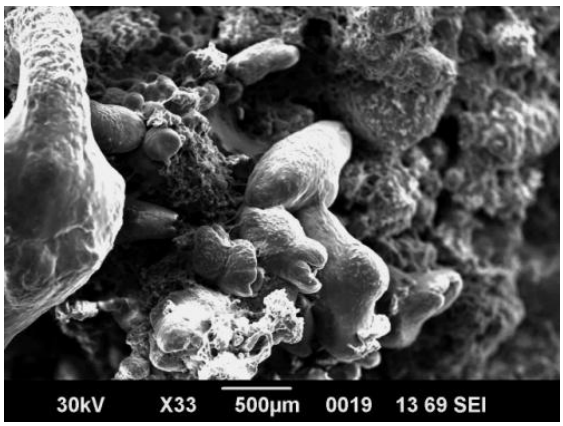
Picture 12. The electron microscopy photograph shows embryonic callus clumps-shaped stage embryos, bar = 500 µm



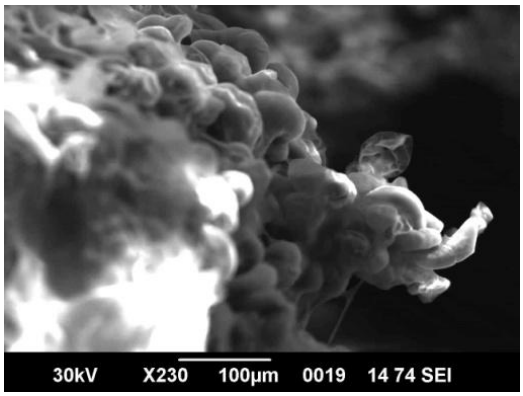
Picture 13. The electron microscopy photograph shows embryonic callus clumps -shaped stage embryos, bar = 1.00 mm



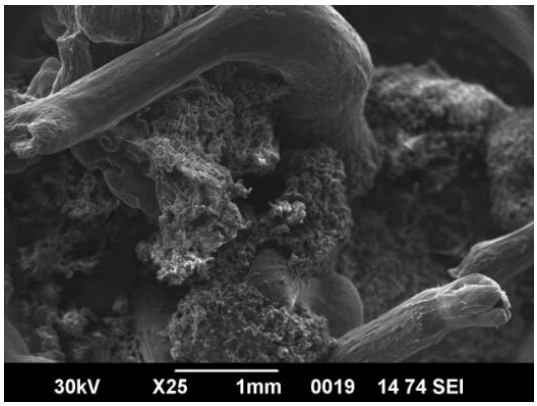
Picture 14. The electron microscopy photograph shows Cotyledon-shaped stage embryos, bar = 200 µm.



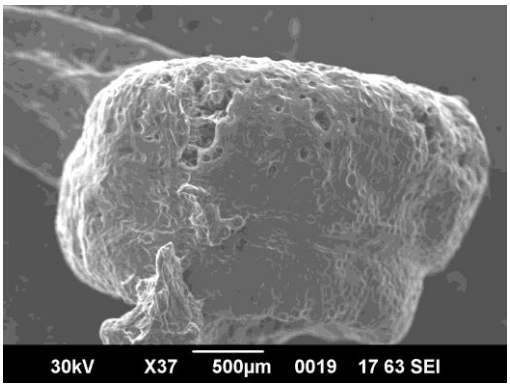
Picture 15. The electron microscopy photograph shows Cotyledon-shaped stage embryos, bar = 500 µm



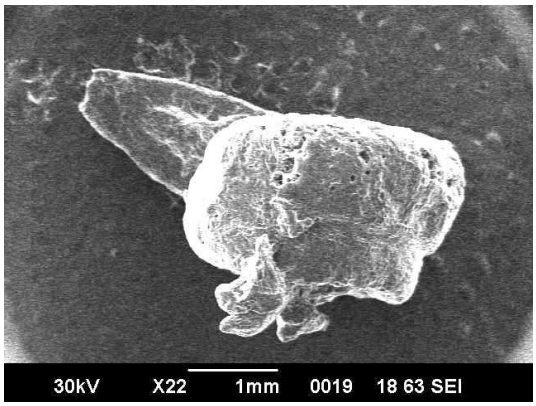
Picture 16. The electron microscopy photograph shows Torpedo-shaped stage embryos, bar = 100 µm



Picture 17. The electron microscopy photograph shows Torpedo-shaped stage embryos, bar = 1.00 mm



Picture 18. The electron microscopy photograph shows the embryonic callus induction procedure (after 45 days) of cultivation on a hormone-free MS medium showed that the embryonic callus did not form. , bar = 500 µm



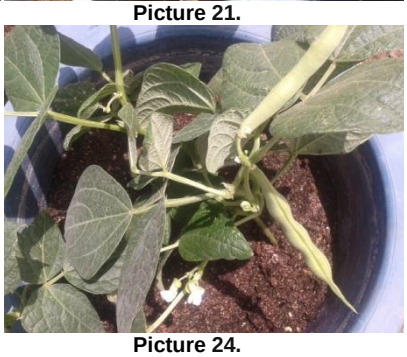
Picture 19. The electron microscopy photograph shows the embryonic callus induction procedure (after 45 days) of cultivation on a hormone-free MS medium showed that the embryonic callus did not form, bar = 1 mm

Picture 20.

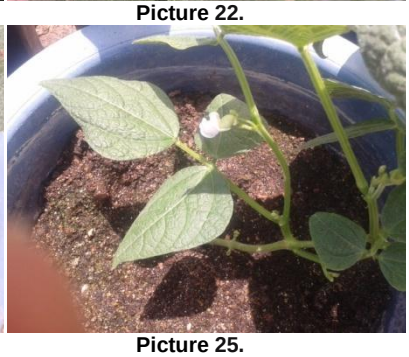
Picture 21.

Picture 22.

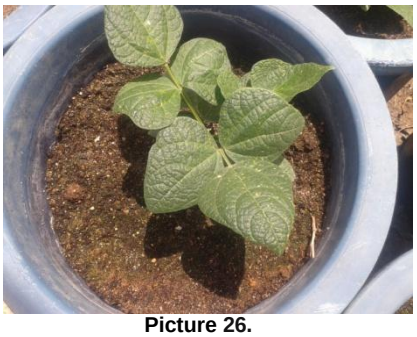
Picture 23.



Picture 24.



Picture 25.


Picture 26.


Picture 27.


Picture 28.

Pictures 20-28. The photographs show the regeneration of old common bean seeds in pots medium, where they developed into healthy plants

5. CONCLUSION

To determine which techniques are most successful for regenerating aged seeds, it is critical to compare them. This study established that prolonged hypoxia throughout a protracted period of storage can inhibit germination. While hydrogen peroxide treatment (20 mM) supplies oxygen throughout the germination process, it can overcome hypoxia during bean seed imbibitions and germination. Catalase enzyme (10.5 units/mL) and other catalytic agents, such as transition metal ions like Fe^{+2}, Cu^{+2}, and Mn^{+2} are crucial for converting hydrogen peroxide into oxygen., transition metal ions can be used at the following concentrations: 0.001M, 0.001M, and 0.0001M, respectively. A protective layer of $CaSO_4$ (0.5 mM) was applied since aged seeds have very fragile biological membranes. The test of trazolium gave results for some common bean seeds that it is non-viable or some parts of the seed are damaged or defective; However, 2,4-D at a concentration of 5 mg/l + supplements was able to produce somatic embryos of the old seeds tissues for common bean cv. Giza 6 (a local cultivar).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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