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N^6-benzyladenine (BAP)-Based Seed Preconditioning Enhances the Shoot Regeneration of Seedling-Derived Explants for Subsequent Indirect Gene Transfer in Soybeans (*Glycine max* [L.] Merrill.)

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Abstract: This study evaluated the effects of N^6-benzyladenine (BAP) seed preconditioning and seedling-derived explants on in vitro plant regeneration potential in soybeans (*Glycine max* [L.] Merrill.). The findings showed that seed preconditioning with 2.55 mg/L BAP prior to germination significantly influenced seedling establishment and the development of shoots, shoot elongation, and rooting on MS media supplemented with BAP and TDZ, compared to the negative (MS-NC) and positive (MS-NP) controls. The results also showed significant differences based on the genotypes, with Dundee recording 91.0% germination over a minimum of 5 days, compared to 74.2% with Peking, followed by 87.5% and 80.0% overall shoot induction frequency in these genotypes, respectively. Regenerated shoots were successfully elongated on MS medium supplemented with 0.5 mg/L BAP plus 0.6 mg/L GA_3 and rooted on hormone-free medium, for 3-4 weeks, and then hardened in the acclimatization growth room under elevated light levels. Overall, this study revealed that BAP preconditioning of seeds enhances the frequency of bud initiation and shoot proliferation, mostly in whole-seedling and cotyledonary node explants subcultured on MS-E and MS-A media supplemented with BAP in combination with TDZ.

Keywords: N^6-benzyladenine; cotyledonary nodes; half-seeds; gibberellic acid; MS medium; shoot induction; thidiazuron; rooting; whole-seedling explants

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1. Introduction

Glycine max [L.] Merrill., the soybean, serves as one of the most important industrial legumes belonging to the Fabaceae family. Preferably, this crop is used as a grain or pulse due to its high-quality proteins, vegetable oil, carbohydrates, fiber, vitamins, and mineral nutrients, which are required for the manufacturing of many health and nutritional products, as well as in various industrial applications [1]. Soybeans, like many other legumes, are central to sustainable agriculture, nutrient cycles, human nutrition, and food security. However, the genetic improvement of the soybean against its narrow gene pool using modern crop improvement techniques such as genome editing [2] and *Agrobacterium tumefaciens*-mediated genetic transformation [3] still present many hurdles in the development of newly improved fertile progenies through direct or indirect organogenesis via plant tissue culture. Although fertile transgenic soybean plants have been regenerated in vitro [1,4–6], ongoing research still needs to be accelerated on the establishment of an efficient in vitro plant regeneration system, a prerequisite for the successful application of CRISPR-based genome editing, chemically induced mutagenesis, and *A. tumefaciens*-mediated transformation for improved breeding of soybeans and other agronomically valuable non-legumes and leguminous crops. Plant regeneration coupled with transformation in soybeans is mostly conducted through the cotyledonary node system, but this approach is prone to the production of chimeras, as earlier alluded to by

Jordan and Hobbs [7], it is highly genotype-specific, and the regeneration protocol of transgenic plantlets is usually irreproducible. A highly efficient genotype-dependent somatic embryogenic regeneration protocol has only been reported in the soybean cultivar Jack, which was also considered to be of inferior agronomic value [8]. As a result, studies that continue to explore the regeneration potential of different genotypes, explant types, and culture media compositions are still encouraged. Begum et al. [5] reported a 100% multiple shoot regeneration from cotyledonary nodal segments using Murashige and Skoog (MS) [9] medium supplemented with N^6-benzyladenine (BAP), and BAP plus 1-naphthalene acetic acid (NAA) in the soybean BARI-5 variety. In another study, seven commercial varieties of soybean (Bohemians, Cardiff, Gallen, Merlin, Moravians, Naya, and Silensia) were efficiently regenerated using half-seed explants initiated on media containing BAP and GA_3 after 2-3 weeks of culture [10]. This clearly demonstrates that factors such as the explant choice, type of culture medium, and plant growth regulators (PGRs) play a key role in influencing the regenerative capacity of in vitro cultured tissues in soybeans. Among the PGRs, BAP serves a critical role in plant tissue culture as a key complex compound produced through a de novo biosynthesis as ribosides, nucleosides, and/or nucleotides [11]. In this study, we therefore evaluated the effects of BAP during both seed preconditioning and efficient shoot culture establishment with the inclusion of thidiazuron (TDZ) to enhance the regeneration capability of half-seed, single-cotyledonary-node (coty-node), double-coty-node, and whole-seedling explants in two soybean cultivars: Dundee and Peking. Thus, our study highlights seed preconditioning with BAP as a newly optimized approach to improving the quality, amenability, and potency/vigor of seedlings produced as an explant source for efficient in vitro shoot induction and subsequent regeneration during *A. tumefaciens*-mediated genetic transformation in soybeans.

2. Materials and Methods

2.1. Plant Materials and Culture Medium

The seeds of two soybean (*Glycine max* [L.] Merrill.) cultivars, Dundee and Peking (Figure 1A,B), collected from Amaloba Nursery at the Turfloop Campus, University of Limpopo, South Africa, were used in this study. The seeds were freshly harvested, stored in brown paper bags, and kept under room temperature until use. For experimental purposes, the seeds were surface-decontaminated using chlorine gas according to a modified protocol, as described by Mangena and Mokwala [12]. The seeds were initially washed with running tap water with a detergent to remove dust and other soil detritus. After washing, the seeds were rinsed a few times with sterile distilled water (dH_2O) and placed on Whatman grade 1 (110 mm) filter papers for drying at room temperature. Dried seeds were then transferred into 100 mm Petri dishes and sterilized for 16 h using chlorine gas generated in a desiccator jar by mixing 100 mL of 3.5% (1:10 *v*/*v*) sodium hypochlorite (domestic bleach) with 4.0 mL of hydrochloric acid (HCl).

Figure 1. Soybean seeds (*Glycine max* L. Merrill.): Dundee (**A**) and Peking (**B**) cultivars. Representations of Dundee half-seed explants (**C**) prepared after 3 h of seed preconditioning in 2.55 mg/L BAP, and

seedling-derived Peking whole-seedling (**D**), Dundee double-coty-node (**E**) and Peking single-coty-node (**F**) explants prepared after 10 days of incubation in a tissue culture growth room.

2.2. Preparation of MS Culture Media

The Murashige and Skoog (MS) basal culture medium was used in this study. The culture medium was prepared as described by Murashige and Skoog [9], comprising 3% sucrose and 0.3% gelrite, with the pH adjusted to 5.8 using 0.1 N HCl or NaOH. However, the different compositions and types of plant growth regulators (PGRs) were modified according to the purpose of the culture with the inclusion of cytokinin N^6-benzyladenine (BAP) in combination with thidiazuron (TDZ), and gibberellic acid (GA_3) for shoot induction and elongation, respectively. MS basal culture media were supplemented with different compositions of BAP and TDZ, denoted as MS-A (2.00 mg/L BAP), MS-B (2.55 mg/L BAP), MS-C (1.22 mg/L TDZ), MS-D (1.22 mg/L BAP plus 0.5 mg/L TDZ), and MS-E (0.5 mg/L BAP plus 1.22 mg/L TDZ), and used for shoot proliferation and multiplication. The MS culture media for shoot induction used as the negative (MS-NC) and positive (MS-PC) controls were both not supplemented with PGRs. For in vitro elongation and rooting, MS media supplemented with 0.5 mg/L BAP plus 0.6 mg/L GA_3, and hormone-free medium, were used, respectively. All chemical compounds used for the preparation of the MS culture medium and PGR stock solutions were purchased from Lasec (Pty) Ltd. (Cape Town, South Africa) and Merck (Sigma-Aldrich, Midrand, South Africa), respectively. The media were autoclaved at a 121 °C temperature with 1.00 kg/cm^2 pressure for 20 min and distributed into 150 baby-jar culture vessels.

2.3. Seed Preconditioning, Germination, and Seedling Establishment

The disinfected soybean seeds were imbibed in 2.55 mg/L BAP solution for 3 h with gentle agitation (150 rpm) using an OrbiShake platform (Labotec, Midrand, South Africa). Seeds used as a control were imbibed in sterile distilled water for the same duration. After imbibition, the preconditioned and control seeds were rinsed a few times with sterile distilled water to remove excess BAP solution from the seed surfaces, and then placed in presterilized Petri dishes (Lasec, SA), double-layered with moistened sterile Whatman no. 1 filter papers (Prestige Chemicals, Durban, South Africa) were used for germination. All seed cultures were kept in a culture room under controlled environmental conditions, and the final germination response was assessed as the growth of the radicle and epicotyl, leading to well-established seedlings. This was determined using the equation $G = G_N/N$, multiplied by 100%. In this case, G, GN, and N refer to the final germination percent, total number of germinated seeds, and number of cultured seeds after 10 days of incubation in the growth room. Cotyledonary node (double- and single-coty-node) and whole-seedling explants were prepared from the germinated seeds. Furthermore, the BAP preconditioned seeds used to develop the half-seed explants were not incubated for germination but were directly used for explant preparation, as described below.

2.4. Explant Preparation and Subculturing for Shoot Induction

The seedlings developed from the BAP-pretreated and control soybean seeds were used to prepare double- and single-coty-node explants (Figure 1E,F) by excising off the epicotyls and hypocotyls (4-6 mm beneath the cotyledons). Whole-seedling explants (Figure 1D) were prepared by slightly trimming the roots and removing the epicotyls, as described in our previous study [13]. The half-seed explants (Figure 1C) were, however, prepared immediately after a 3 h seed imbibition (both the BAP-preconditioned and control seeds), by removing the seed coat and the embryo and splitting the cotyledons [14]. All seed and seedling-derived soybean explants were subcultured for 3–6 weeks on different MS media (MS-A, MS-B, MS-C, MS-D, MS-E, MS-NC, and MS-PC) for shoot proliferation and multiplication. For the preparation of the MS-NC and MS-PC shoot cultures, the different types of explants were prepared from soybean seedlings developed from seeds

preconditioned in sterile dH_2O and 2.55 mg/L BAP, respectively, cultured on MS media without PGRs, prepared as indicated in Section 2.2.

2.5. Shoot Elongation, Rooting, and Acclimatization Ex Vitro

All shoots developed from the different MS media were excised from explants and cultured for elongation on MS basal culture medium supplemented with 0.5 mg/L BAP and 0.6 mg/L GA_3. For rooting, the elongated shoots were then transferred to MS medium without the addition of PGRs. Elongated individual shoots were immediately subcultured for rooting after reaching 3–6 cm in shoot length. In vitro elongation and rooting cultures were maintained in a culture room for 3-8 weeks. The successfully rooted shoots were transferred to culture vessels quarter-filled with sterile vermiculite and covered with transparent plastic bags. These plantlets were hardened and acclimatized ex vitro in a growth room that was equipped with elevated light intensity, with a 16:8 h photoperiod and ambient relative humidity.

2.6. Growth Conditions, Data Collection, and Statistical Analyses

All in vitro plant regeneration cultures were incubated in a tissue culture growth room at a temperature of 24 ± 2 °C, 60-80 $\mu molm^{-2}s^{-1}$, and with a 16:8 h (light/dark cycle) photoperiod provided by cool white-fluorescent lights under 50-60% humidity. Acclimatization ex vitro was achieved in the acclimatization room with 120-200 $\mu molm^{-2}s^{-1}$ under the same regulated temperature and photoperiod, as well as ambient relative humidity. Data collected included the number of germinated seeds (plus hypocotyl length, epicotyl length, and girth) and the percentage of explants inducing shoot buds, intact shoots, multiple shoots, and elongated as well as rooted shoots. All experiments were repeated thrice with a minimum of 30 explants per experiment. Data were expressed as mean values and analyzed for the statistical mean differences and significance using a One-Way ANOVA with SPSS software version 28.0 (IBM, Chicago, IL, USA).

3. Results

3.1. Effects of BAP Seed Preconditioning on Germination and Seedling Development

Soybean seeds of the cultivars Dundee and Peking were first pretreated with 2.55 mg/L BAP for 3 h before germination in Petri dishes overlaid with a double layer of sterile filter papers moistened with sterile dH_2O. The results indicated that seed germination percentage and seedling growth differed according to BAP seed pretreatment and the genotype/cultivar. As indicated in Figure 2, both the germination percentage (Figure 2A,B), and morphology of the developed seedlings, such as the hypocotyl and epicotyl lengths, as well as the hypocotyl girths (Figure 2C–E), were significantly influenced by the factors mentioned above. Although the seeds started germinating after 2 days of culture, a considerable difference in germination progression was observed daily, as indicated in Figure 2. As per the observations, the Dundee soybean cultivar recorded the highest germination percentage of 91% over a minimum of 5 days (Figure 2A), which proved to be rapid compared to the Peking cultivar. The germination of Peking seeds was gradual, with maximums of 70.1 and 74.2% in both the control and BAP-pretreated seeds, respectively (Figure 1B). Furthermore, the seedlings pretreated with 2.55 mg/L BAP generally presented root growth inhibition, in addition to stunted epicotyls, and thicker hypocotyls compared to the controls, as recorded in Figure 2. The findings obtained in this study showed that primary and lateral root development were also inhibited by BAP when compared to the control seedlings whose seeds were imbibed in dH_2O, which exhibited normal rooting (Figure 2C–E).

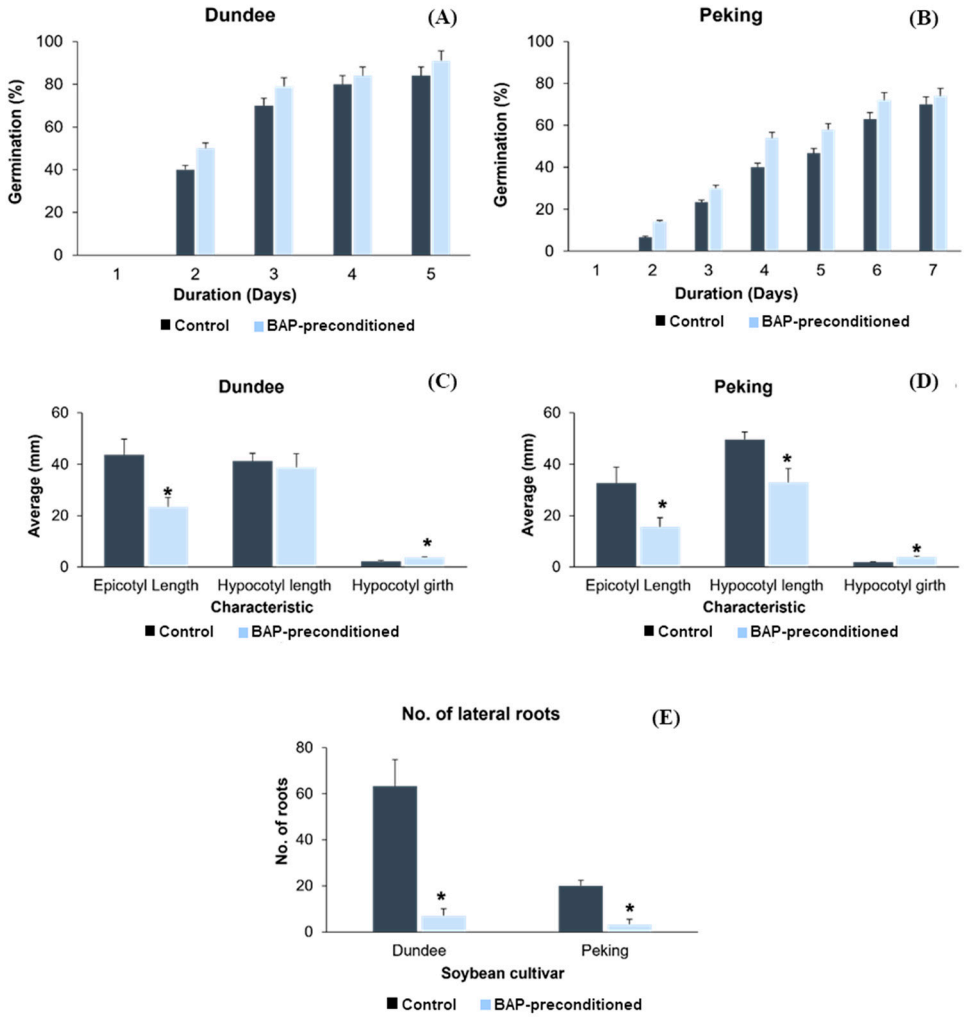

Figure 2. The effect of 2.55 mg/L BAP on seed germination and seedling development in soybean (*Glycine max* L. Merrill.) cultivars Dundee and Peking: (**A**,**B**) present the daily germination percentages recorded after seed imbibition in the 2.55 mg/L BAP solution for 3 h; (**C**–**E**) show variations in root development and hypocotyl and epicotyl lengths and girths recorded after 10 days of growth of the control and BAP-pretreated seedlings. Vertical bars in each SIM treatment with asterisks indicate that mean values were statistically significant at $p < 0.001$.

3.2. Effect of Culture Medium and Explants on Shoot Proliferation

The effect of BAP/TDZ-supplemented basal MS culture medium on shoot regeneration was evaluated using different types of explants prepared from soybean seedlings, established as indicated above. Out of the five media compositions (MS-A, MS-B, MS-C, MS-D, and MS-E), the highest shoot induction frequency was recorded on MS-E, containing 0.5 mg/L BAP and 1.22 mg/L TDZ, with 87.5% in Dundee. This was followed by an average frequency of 83.3% observed in both MS-B and MS-C, as well as 75.0 and 62.5%, respectively, in MS-A and MS-D. In contrast to the control, maintaining the explants derived from BAP-pretreated Dundee seeds on MS-PC culture medium yielded over 41.7% shoot formation frequency, as indicated in Figure 3A. In comparison, MS-E, together with the MS-D medium, recorded a 33.3% shoot induction frequency in Peking (Figure 3A). These were 2.5-fold lower than MS-A, which recorded the highest shoot induction frequency of over 80% compared to all other culture media used for this cultivar (Peking), followed by MS-B, MS-C, and the control (MS-PC) with 66.7, 58.3, and 41.7%, respectively. Overall, MS-A (29.1, 41.7%), MS-PC (29.1, 33.3%), MS-B (12.5, 25.0%), and MS-C (4.2, 16.0%), consecutively, for

Dundee and Peking, produced the best responses for shoot multiplication per explant in both cultivars (Figure 3B).

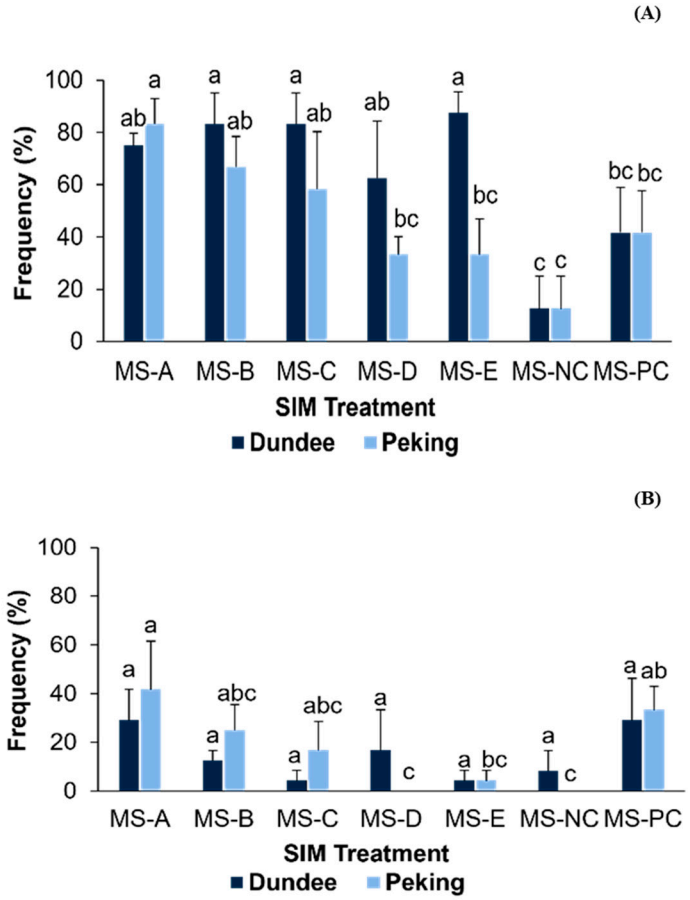

Figure 3. Overall performance of explants regarding the frequency of shoot proliferation (**A**) and shoot multiplication (**B**), determined through the total average (%) of shoots formed per culture medium. Vertical bars in each SIM treatment with different lowercase letters are statistically different at $p < 0.05$.

3.3. In Vitro Elongation and Rooting of Induced Shoots

The rate of shoot elongation, as well as the mean heights of the elongated shoots, also differed based on the genotype and possible habitual effects from the shoot multiplication medium. The Dundee soybean cultivar showed the most efficient shoot elongation response compared to Peking; shoots derived from MS-E exhibited the highest frequency of shoot elongation (Figure 4A). The shoots derived from the MS-PC control medium also showed a considerable shoot elongation capacity of 62.0% for Dundee and 16.7% for Peking. Comparatively, the shoots derived from MS-A, MS-B, MS-C, and MS-D recorded 29.1, 35.1, 31.6, and 16.7% elongation frequencies, significantly lower than MS-E and the positive control (MS-PC). Although Peking showed the lowest frequency of shoot elongation, this cultivar gave the overall tallest shoots compared to Dundee. These shoots also presented a notable difference in the rooting responses of elongated shoots, also based on the culture medium that they were derived from and the genotype (Figure 4B). The highest frequency of rooted shoots was observed from the control (MS-PC in Peking), followed by MS-A and MS-B, with at least 50% rooting frequency that took place within 3 weeks of culture in both cultivars.

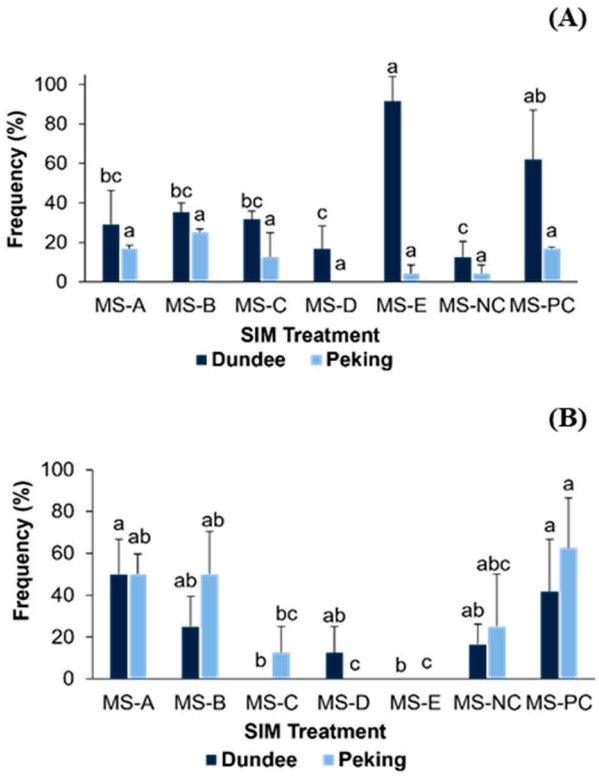

Figure 4. Overall analysis of the frequency of shoot elongation and rooting of individual shoots derived from MS-A, MS-B, MS-C, MS-D, MS-E, MS-NC, and MS-PC. For elongation (**A**), MS culture medium was supplemented with 0.5 mg/L BAP and 0.6 mg/L GA_3, while in vitro rooting (**B**) of the elongated shoots was conducted on a hormone-free MS basal culture medium. Vertical bars in each SIM treatment with different lowercase letters are statistically different at $p < 0.05$.

3.4. Overall Analysis of the Role of BAP on Shoot Regeneration in Soybeans

The overall comparison of the regenerative capacity of the different types of explants and culture media used for shoot regeneration revealed that the BAP-pretreated seedlings were useful for producing the half-seed, single-coty-node, double-coty-node, and whole-seedling explants used in this study. Further, a considerable number of adventitious shoots were formed from these explants (as exemplified in Figure 5), which were efficiently elongated on MS medium supplemented with 0.5 mg/L BAP and 0.6 mg/L GA_3, followed by in vitro rooting on PGR-free MS medium. Apart from BAP, a cytokinin, and TDZ, a phenyl urea derivative with cytokinin-like activity, the evaluated in vitro regeneration capacity of the different explants suggested that the explant type, in addition to the BAP-containing basal MS culture medium and the genotype factor, was responsible for the variations observed in the number of shoots induced per explant, followed by the elongation and rooting of those in vitro developed shoots. The observations further suggested that in vitro shoot induction responses also differed according to the concentrations and combinations of BAP and TDZ, with a considerable level of synergy that occurred among the PGRs. Following the observations made per treatment, the BAP-containing cultures gave a better shoot regeneration response compared to TDZ alone. When TDZ was tested for seed preconditioning (results not shown), very delicate and weak seedlings were produced. However, in general, the findings also suggest that the combination of BAP and TDZ did not exert a significant promotive effect on shoot regeneration frequency compared to BAP alone, especially in the coty-node and half-seed explants. As observed in Figures 3 and 4, the two cultivars responded differently when BAP and TDZ were combined; shoot formation was comparably influenced, particularly in the whole-seedling and half-seed explants, in both soybean cultivars.

Figure 5. Examples of in vitro shoot induction and plantlet regeneration achieved on MS medium supplemented with BAP/TDZ. (**A**) Multiple adventitious shoots induced on MS-E in Dundee whole-seedling explants after 4 weeks of shoot culture, (**B**) Peking single-coty-node explants cultured on MS-E medium after 4 weeks of shoot induction, (**C**) Peking double-coty-node explants cultured on MS-A after 4 weeks of shoot induction culture, and (**D**) Dundee negative control double-coty-node explants after 4 weeks of shoot culture, and a 9-week-old regenerated Dundee soybean plant potted and covered with transparent plastic during the acclimatization stage (**E**).

Moreover, as indicated by the frequency of shoot induction, the explant type and genotype had a significant influence on the regenerative responses observed in this study. According to the observations, shoot bud formation occurred within the first week of culture in the single-coty-node, double-coty-node, and whole-seedling explants, but not the half-seeds, which took about 4 weeks to initiate proliferative shoot buds. Although the half-seed explants showed delays on shoot bud initiation, the overall analysis in Figure 6 demonstrates that this explant produced the highest number of shoots per explant, with about 9.6 shoots being proliferated on average, followed by the whole-seedling and cotyledonary node explants, as illustrated in the same figure.

The frequency of shoot bud formation, displayed in Figure 6, also showed that the response of the half-seed explants at 78% was significantly higher than the 73.3% and 71.4% observed for the whole-seedling and double-coty-node explants, respectively. The single-coty-node explants recorded the lowest number and frequency of shoot buds compared to all types of seedling-derived explants. Although the Peking explants were able to induce shoot buds, their response from the double-coty-node and half-seed explants was much lower than that of the single-coty-node and whole-seedling explants, as well as compared to their Dundee counterparts. As indicated in Figure 6A–D, the regenerative capacity of single-coty-node (47.6%) and double-coty-node (49.5%) explants to form shoots was significantly different from whole-seedling (73.3%) and half-seed (27.1%) explants. The highest number of shoot buds per explant in Peking was obtained from the whole-seedling explants, which recorded a total of 10.5 shoot buds, as indicated in Figure 3C. However, the proliferation of shoots from these buds per explant was somewhat reduced across all explant types as the number of distinct shoots formed per explant were significantly reduced from the total number of formed buds. The whole-seedling explants produced 57.9 and 45.2% multiple shoot induction frequencies in both Dundee and Peking, respectively. This was

47–60% higher than the observations made in single and double-coty-node explants in both genotypes. Furthermore, the Dundee whole-seedling explants produced more shoots than the half-seeds, which initially gave the highest number of shoot buds, followed by the 1.4- and 0.6-fold shoot numbers per explant recorded in the double- and single-coty-node explants. A similar trend was also observed for Peking, in which the half-seed explants showed twice-less the number of shoots from their high number of initiated shoot buds than the 1.9- and 0.6-fold shoot numbers observed in the double- and single-coty-node explants (Figure 6C,D).

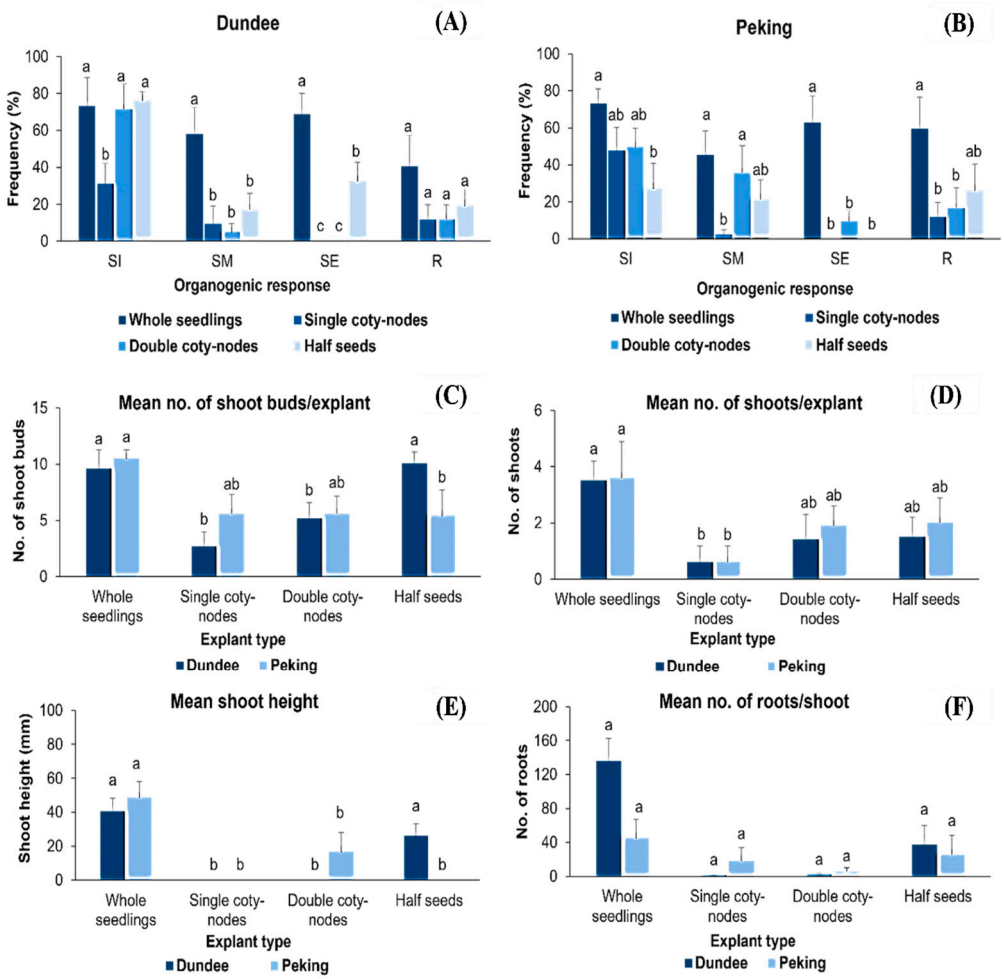

Figure 6. Graphical representation of the organogenic responses of various soybean explants. The overall frequencies of shoot initiation (SI), shoot multiplication (SM), shoot elongation (SE), and rooting (R) in Dundee (**A**) and Peking (**B**) explants. Demonstration of the average number of shoot buds (**C**) and distinct shoots (**D**), the length of elongated shoots (**E**), as well as the number of roots formed per explant in cv. Dundee and Peking (**F**). Mean values marked with the same letters above bars (for each cultivar) are not significantly different according to Fisher's Least Significant Difference (LSD) test at the 5% confidence level.

4. Discussion

Seed germination is considered the most crucial stage for seedling and plant establishment [15], and the success of in vitro regeneration is highly dependent on this stage of culture. As the findings reported in this study demonstrated, variations in gemination and seedling development occurred as a result of the genotype and BAP, which caused notable differences in the germination rates and morphology of the established seedlings. This dual BAP–genotype variability was also reported by Raza et al. [4], wherein nine

soybean genotypes gave significantly different germination outcomes, and the inclusion of cytokinins during seed cultures also resulted in distinguished effects on the morphology of seedlings, such as enlarged cotyledons and short and thick hypocotyls, as observed in this study. Overall, all soybean seedlings treated with BAP presented stunted and fewer roots and shorter epicotyl and hypocotyl lengths, as described by Raza et al. [4], and as shown in Figure 2C–E. Walne et al. [16] also reported maximum germination and different seedling growth properties following a comparison among different corn, cotton, and soybean genotypes. Their study successfully reported cumulative seed germination and seedling establishment that differed according to the genotype, with the growth relationships being accurately described with bilinear functions for the corn and cotton, as well as a quadratic function for the soybeans.

As our results have shown, MS-A (29.1, 41.7%), MS-PC (29.1, 33.3%), MS-B (12.5, 25.0%), and MS-C (4.2, 16.0%), consecutively, for Dundee and Peking, produced the best responses of shoot multiplication in both cultivars (Figure 3B). These findings also differed according to the genotype and culture media (containing BAP and TDZ) in terms of the frequency of multiple shoot formation per explant. Similar findings were reported by Phat et al. [17] on the regeneration of several Korean soybean cultivars. Their study reported that high frequencies of both germination and shoot induction relied upon the composition of the culture medium, in addition to restricted dormancy, which all influenced in vitro regeneration in the soybeans. Nevertheless, our results showed similar observations of shoot induction frequencies that were significantly different according to both the composition of the MS medium and the genotype (Figure 3A). The ability of shoot buds to proliferate into distinct shoots was monitored throughout the period of culture for the two soybean cultivars. Assessing the mean number of multiple shoots induced per explant in Dundee also revealed that significant differences were observed when BAP or TDZ were used alone rather than in combination in a culture medium (MS-A to MS-E). PGRs such as BAP and TDZ have been used previously to initiate cell division and differentiation to form multiple shoots in soybean cell cultures [4,5,18]. As indicated in Figure 3B, the MS culture media supplemented with 2.00 mg/L BAP (MS-A), 2.55 mg/L BAP (MS-B), and 1.22 mg/L BAP in combination with 0.5 mg/L TDZ (MS-D), like MS-C, gave the highest frequency of induced multiple shoots, at 41.7, 25.0, and 16.7%, respectively.

Nevertheless, the latter gave a multiplication frequency like that of the MS-PC medium used as a positive control. Although the MS-C, MS-D, and MS-E culture media stimulated considerably high numbers of shoots, the morphology of some of the induced multiple shoots exhibited stunted and abnormal morphology, such as shoot fasciation and unproportionately enlarged leaves. The high frequency of emerging shoots in MS-A compared to MS-C and other PGR-containing media shows that the MS medium composition with 2.0 mg/L BAP used in this study and other previous studies still serves as an excellent combination for the establishment of in vitro regeneration cultures in soybeans. In addition, assessing the different MS media utilized also demonstrated that high frequencies of shoot formation were found on medium containing BAP, followed by TDZ in combination with this hormone. This also clearly implied that these hormones plus MS basal culture medium positively influenced shoot proliferation in soybeans, as reported by Bhattacharya et al. [19] and Damanik et al. [20]. Furthermore, rooting frequencies were significantly diverse among the shoots derived from different media treatments, and this was not largely based on differences in the periods taken for root induction, as suggested by Begum et al. [5]. For Dundee, the highest root formations were obtained from MS-A, MS-PC, MS-B, MS-NC, and MS-D, at 50, 41.7, 25, 16.5, and 12.5%, respectively. The shoots derived from MS-C and MS-E took over 2 weeks before they showed root formation, including the Peking shoots derived from the MS-D culture medium. Furthermore, more than 70% of the rooted plantlets were successfully acclimatized in the greenhouse, irrespective of the genotype and regeneration media. This occurred even though it was clear that the culture media and composition influenced the elongation and root induction capacities of the elongated shoots. Like a few previously reported studies [20,21], in vitro rooting was not affected by shoot regeneration

cultures, and none of the cultures also influenced the plantlets' acclimatization and hardening under ex vitro environmental conditions. Preferably, the inclusion of IBA and NAA is recommended to work better during root proliferation in most in vitro cultures, which clearly demonstrated positive effects on the survival of regenerated plants ex vitro [22–24].

However, the overall results indicated that efficient plant regeneration and acclimatization was achieved using the BAP-initiated cultures, as reported by Polisetty et al. [25], Soto et al. [26], and Raza et al. [4] in soybeans. Related studies in cowpeas (*Vigna unguiculata* L. Walp.), lentils (*Lens culinaris* Medik.), and various other *Phaseolus* spp., *Pisum* sp., *Cicer* spp., and *Vigna* spp. samples also alluded to the efficient role of BAP, and then TDZ, on the in vitro regeneration of plants, as reported by Sindhu et al. [27], Khabbazi et al. [28], and Pratap et al. [29]. Also, the overall comparison of the explants, mainly the single-coty-node, double-cotyledonary-node, and seedling explants, initiated shoot formation in the second week of culture, unlike the half-seed explants. Similar observations were also made by Paz et al. [14] and Zia et al. [30] in various soybean cultivars (Thorne, Williams, Williams79, Williams82, NARC-4, and NARC-7), respectively. However, in line with their observations, and like the seed germination and seedling growth observed in this study, the Dundee cultivar showed the most effective bud initiation, shoot induction frequency, and capacity of plant regeneration compared to Peking in all cultures. While significant progress has been achieved in the tissue culture of many soybean genotypes [1,5,10,12,17], an efficient regeneration protocol for use during indirect gene transfer methods is still yet to be obtained. Generally, the lack of a more reproducible, efficacious, and genotype-independent regeneration protocol remains a major challenge for many laboratories. Therefore, BAP-based seed preconditioning prior to germination and explant preparation from pretreated seedlings will overcome the unwanted habitual effects of the hormone, enhancing the quality of the explants and potentially reducing the severity of *Agrobacterium*-induced tissue senescence in in vitro cultures [12,14].

5. Conclusions

The present findings conclude that seedling preconditioning with BAP enhanced the frequency of bud initiation and shoot proliferation, mostly when whole-seedling and cotyledonary explants were subcultured on MS-E and MS-A culture media supplemented with BAP and TDZ. The results reaffirm the role of BAP, in addition to TDZ, in promoting the rapid and efficient in vitro regeneration of plants in soybeans and other leguminous, as well as non-leguminous, crop species. These findings further suggest that soybeans can be efficiently regenerated using cotyledonary-node (single- and double-coty-node) and whole-seedling explants in particular for subsequent mutagenic breeding and *Agrobacterium tumefaciens*-mediated genetic transformation under in vitro plant cell culture conditions.

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