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Comparison of *in vitro* Regeneration Potential of Different Preconditioned and Nonconditioned Explants of Peanut (*Arachis hypogaea* L)

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Abstract: The study presents the *in vitro* regeneration potential of three different explants of peanut. The explants used were zygotic embryos, plumular apices and the embryonic axis of peanut taken after 15 days preconditioning with 25 mg/L Benzylaminopurine (BAP) or nonconditioned on MS (Murashige and Skoog) medium without containing BAP. Subsequently, all preconditioned and nonconditioned explants were cultured on MS medium containing 1 mg/L BAP for multiple shoot induction. Both preconditioned and nonconditioned explants induced 100% shoot regeneration frequency. Shoot counts were highest from preconditioned explants, and shoot length was attributed to nonconditioned explants. Comparison of explants revealed the insignificant impact on shoot counts. However, longer shoots were achieved from zygotic embryos explants. The comparative analysis of preconditioned and nonconditioned explants revealed the highest shoot counts form preconditioned plumular apices explants (25.80) and longer shoots from nonconditioned zygotic embryo explants (4.23 cm). *In vitro* regenerated shoots from preconditioned and nonconditioned explants were successfully rooted, followed by successful adaptation of rooted plantlets in the pots. The results suggest that both preconditioned and nonconditioned explants of peanut can be used for *in vitro* regeneration.
Keywords: *In vitro*, regeneration, peanut, preconditioned, nonconditioned.

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1. Introduction

Peanut (*Arachis hypogaea* L) is a cultivated edible legume crop of the subtropical regions (Hassan et al., 2013). It is an essential source of oil (Suchoszek-Lukaniuk et al., 2011; Tuberoso et al., 2007), biologically active compounds and raw material for various industries (Blomhoff et al., 2008, Özkan and Aasim 2019). Other essential macromolecules include protein, amino acids (Pelto and Armar-Klemesu, 2015), lipids and carbohydrates (Settaluri et al., 2012). These macromolecules play a vital role in human nutrition and curing against several disorders and diseases (Matilsky et al., 2009; Pelkman et al., 2004). The consumption of peanut is highly variable, ranging from raw, roasted, coated with caramels and as peanut by-products all over the World (Varela and Fiszman, 2011; Campos-Mondragón et al., 2009).

Peanut is an important cultivated crop of Turkey, and there is always need to develop elite cultivars to cope up the challenges faced by plants under field conditions (Baloch et al., 2010; Stalker, 2017). Application of biotechnological tools like plant tissue culture offers an alternative and rapid way of developing new elite cultivars in a relatively short time compared to other techniques (Ali and Ray, 2018; Comino et al., 2019; Mitra and Gantait. 2020). However, development of reliable and repeatable *in vitro* regeneration protocol for whole plant regeneration in legumes are generally considered as uphill task due to their recalcitrant nature (Pratap et al., 2018; Raza et al., 2017). Peanut is also considered

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as one of the recalcitrant legume plants towards *in vitro* regeneration. To date, a large number of *in vitro* regeneration protocol have been reported with variable success, low regeneration and complex procedures (Memon et al., 2013; Tiwari and Tuli, 2009; Matan and Prakash 2007). Development of reliable and repeatable protocol is direly needed to develop a new protocol with high regeneration, followed by the development of rooting and acclimatization.

In recent years, researchers are using a different approach for inducing high regeneration frequency of various crops (Otiende and Maimba, 2020). Among these techniques, preconditioning of explants to a higher concentration of cytokinins followed by culture of explants at low cytokinin concentrations either used singly or with any auxin like Indole-3-butyric acid (IBA) or Naphthalene Acetic acid (NAA). Some of the studies on preconditioning of different explants to different cytokinins like BAP (Day et al. 2017; Isah, 2020; Özkan and Aasim, 2019) 2ip (2-isopentyl adenine) or TDZ (Thidiazuron) (Barpete et al. 2014) for variable concentration and exposure time ranging from few days to few weeks. On the other hand, selection of proper explant is also highly significant for the establishment of a new and efficient protocol for gaining healthy shoots or plantlets without showing any abnormal growth or exhibiting any somaclonal variation. Keeping in view, the present study was designed to investigate the *in vitro* regeneration potential of three different preconditioned and nonconditioned explants of peanut cv NC-7 for future work related to applying other biotechnological techniques.

2. Materials and Methods

The seeds of Peanut CV NC-7 in shelled form were procured from Western Mediterranean Agricultural Research Institute (BATEM) Antalya, Turkey. The seeds were isolated from shells manually, and uniform seeds without exhibiting any mechanical damage were separated for sterilization followed by isolation of zygotic embryos (ZE) by following the protocol established by Özkan and Aasim (2019). The ZE explants were detached carefully from seeds and immediately inoculated on MS (Murashige and Skoog, 1962) medium augmented with 25 mg/L BAP for 15 days (preconditioned explants). On the other hand, ZE explants were also inoculated on MS medium devoid of BAP (nonconditioned explants).

After 15 days of initial preconditioning, three different explants; preconditioned zygotic embryos (PZE), plumular apices (PPA) and embryonic axis (PEA) explants were used for *in vitro* regeneration. On the other hand, all these three explants were also isolated from nonconditioned embryos and named as nonconditioned zygotic embryos (NZE), plumular apices (NPA) and embryonic axis (NEA). All these six explants from two different sources were inoculated on MS medium augmented with 1 mg/L BAP for 12 weeks. Thereafter, shoots were isolated from nutrient agar gelled medium and transferred to rooting medium augmented with 1.0 mg/L IBA for six weeks (Özkan and Aasim, 2019). The rooted plantlets were transferred to pots for acclimatization following the protocol established by Özkan and Aasim (2019). Plastic cups were filled with vermiculite having homogenized humidity and covered with polyethene bags were used for acclimatization of rooted plants in the growth room (Day et al., 2017).

The MS basal medium for preconditioning, regeneration and rooting was also augmented with 3% (w/v) sucrose, 0.65% (w/v) agar. The pH of all mediums was optimized at 5.8 and autoclaved (104 kPa, 120 °C temperature, 20 min). All culture mediums were cultured in the growth room provided with white light-emitting diodes (LEDs) with 16/8 h light/dark conditions and temperature (24±2 ° C) were maintained.

At the end of the 12 weeks, data about shoot counts and shoot length (cm) were tabulated and analyzed. Data for callus induction (%) and shoot regeneration (%) were not analyzed due to the 100% response from all explants. *In vitro* regeneration experiment had three replicates with eight explants per replicate. The experimental data were analyzed by applying One Way ANOVA (analysis of variance) with the aid of SPSS21 for Windows (SPSS Inc. Chicago, IL, USA). For means comparison, Duncan's Multiple Range Test (DMRT) was used (*p0.01* significance level). The data were arranged to arcsine ($\sqrt{X}$) transformation (Snedecor and Cocharan, 1967) ahead of ANOVA analysis.

3. Results

The present study enlightens the successful use of three different preconditioned and nonconditioned explants of peanut, followed by culture on medium augmented with a low concentration of BAP.

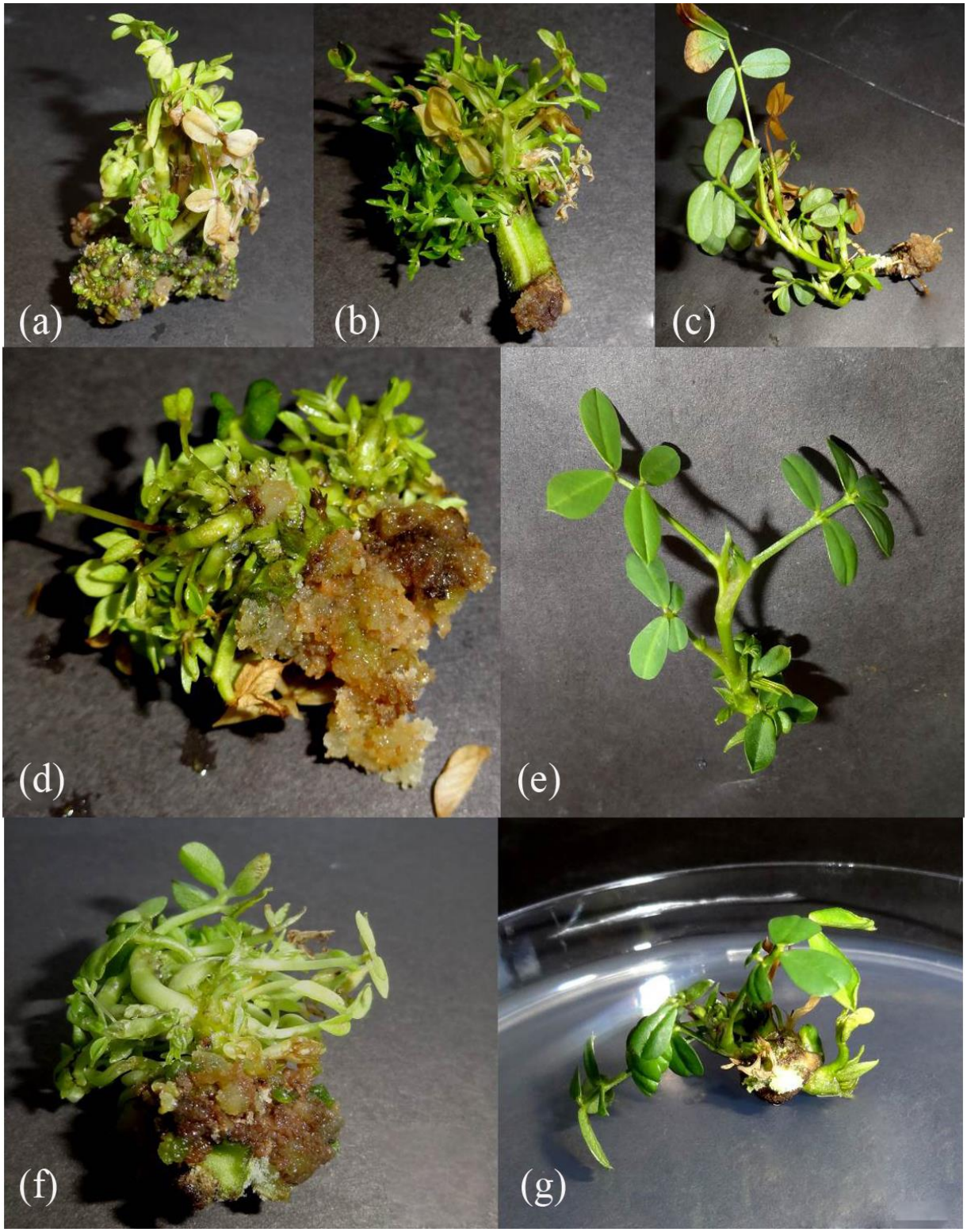

Fig. 1. *In vitro* regeneration from preconditioned and non-conditioned explants of peanut (*Arachis hypogaea* Linn). (a,b) PZE (preconditioned zygotic embryos) with more callus, stunted shoots with small leaf size, (c) NZE (nonconditioned zygotic embryo) with normal growth and low callus, (d) PPA (preconditioned plumular apices) with callus, stunted shoots with small leaf size, (e) NPA (nonconditioned plumular apices) with normal growth, low callus, (f) PPA (preconditioned plumular apices) with callus, stunted shoots with small leaf size, (g) NEA (nonconditioned embryonic axis) with normal growth and low callus.

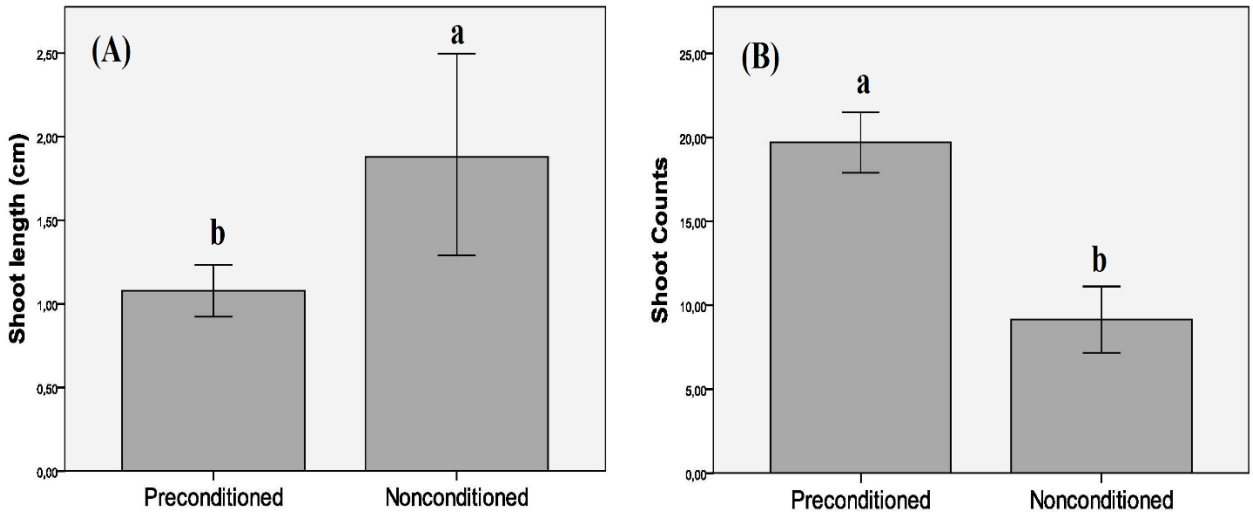

Fig. 2. Impact of preconditioning and nonconditioning on *in vitro* regeneration of peanut, shoot length (A), shoot count (B).

All explants (preconditioned or nonconditioned) exhibited 100% callus and regeneration induction. The callus induction was relatively more on preconditioned explants (Fig 1a,c,e) compared to nonconditioned explants (Fig 1b,d,f). The results of this study also reveal 100% regeneration from all preconditioned and nonconditioned explants.

Comparison of preconditioned and nonconditioned explants exhibited their impact on shoot counts and shoot length in the opposite direction. Shoot counts from preconditioned explants were almost double and recorded 19.69 compared to nonconditioned explants that were recorded 9.14 (Fig 2A). The shoot length was opposite and preconditioning with higher BAP concentration significantly hindered the shoot length and recorded as 1.08 cm compared to 1.88 cm from nonconditioned explants (Fig 2B). Although preconditioned explants induced more shoots, however, it exerted a negative impact on plant growth and resulted in stunted shoots with small leaf size and area irrespective of explant type (Fig 1 a,b,c,e). On the contrary, normal shoots were recorded from nonconditioned explants.

Results on explants source revealed that all explants were highly responsive but exhibited insignificant impact with each other. Shoot counts per explants ranged 12.36-15.76 (Fig 3A) with highest shoot counts were recorded from the embryonic axis (15.76) followed by plumular apices (15.13).

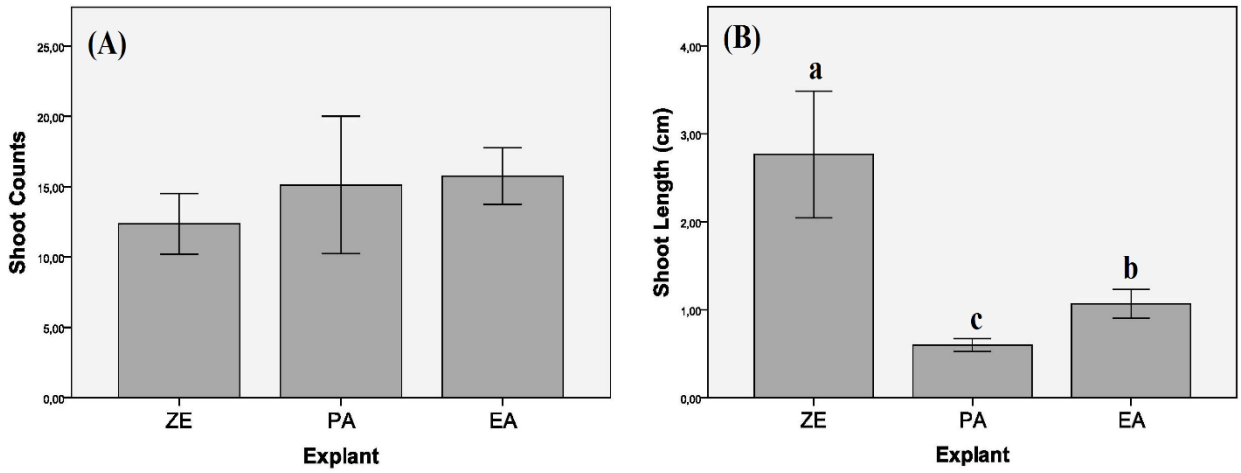

Fig. 3. Impact of different explants on *in vitro* regeneration of peanut, (A), shoot length, (B), shoot count. EA, embryonic axis, PA, plumular apices, ZE, zygotic embryos.

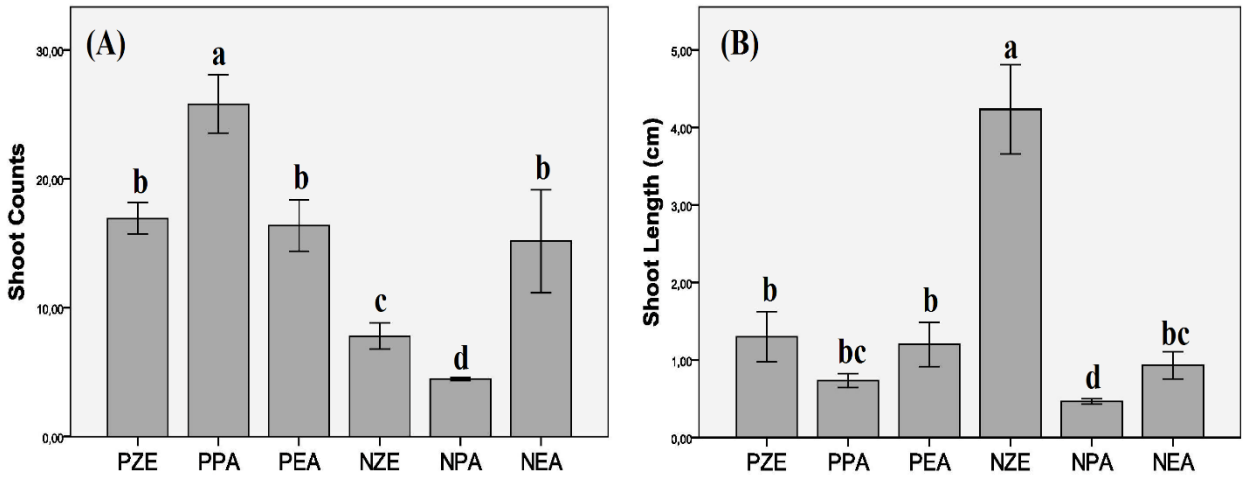

Fig. 4. Impact of combination of preconditioning × explants on *in vitro* regeneration of peanut, (A), shoot length, (B), shoot count. NEA, nonconditioned embryonic axis; NPA, nonconditioned plumular apices; NZE, nonconditioned zygotic embryos; PEA, preconditioned embryonic axis; PPA, preconditioned plumular apices; PZE, preconditioned zygotic embryos.

The minimum shoot counts of 12.36 were recorded on zygotic embryo explants. Results on mean shoot length ranged 0.60 to 2.77 cm with the longest shoots from zygotic embryos and least shorter shoots from plumular apices explants (Fig 3B).

Shoot counts from preconditioned × explants generally generated more shoots compared to nonconditioned × explants. The shoot counts ranged 16.37-25.80 and 4.47-15.15 (Fig 4A) respectively for preconditioned × explants and nonconditioned × explants. Shoot counts from PZE were 2-fold (16.92) compared to NZE (7.80 cm). The response of PPA was recorded 6-fold more shoots (25.80) compared to 4.47 shoots from NPA explants. Whereas, the response of PEA and NEA were statistically at par and recorded as 16.37 and 15.15, respectively. Results on mean shoot length were also affected by the combination of preconditioning × explants and nonconditioning × explants. PPA and PEA explants generated shorter shoots compared to NPA and NEA. Whereas, PZE induced shorter shoots (1.30 cm) compared to NZE (4.23 cm) explants. Among explants, both PZE and NZE explants were most responsive and generated longer shoots of 4.23 cm (NZE) compared to 1.30 cm (PZE) long shoots (Fig 4B). On the contrary, plumular apices were least responsive and generated shorter shoots of 0.47 cm (NPA) compared to 0.73 cm long shoots (PPA). Mean shoot length from embryonic axis explant were recorded as 1.20 cm (PEA) and 0.93 cm (NEA).

In vitro regenerated shoots from both preconditioned and nonconditioned explants were carefully isolated from explants for *in vitro* rooting. These shoots were cultured on medium enriched with 1.0 mg/L IBA. Rooting started from the basal end of the explants within two weeks of culture and shoots were awaited for four weeks before shifting to the pots for acclimatization. For acclimatization, single shoot per pot was covered with polyethene bags for two weeks in the growth room. These plantlets acclimatized well when once bags were removed, and it yielded high acclimatization rate of approximately 70 - 80%.

4. Discussion

Successful establishment of *in vitro* regeneration protocol of recalcitrant plants like peanut is of utmost important (Özkan and Aasim, 2019) and several factors regulate the *in vitro* organogenesis. Among these factors, explants and plant growth regulators are highly significant. One of the strategies includes inoculation of explants with cytokinins (pre-treatment or preconditioning) at relatively higher concentration in order to induce rapid cell division along with enhanced explant size. This technique has been successfully employed for several crop plants like cowpea (Van Le et al., 2002), lentil (Aasim et al., 2012), banana (Madhulatha et al., 2004), Chickpea (Aasim et al., 2011, 2013), grass pea (Barpete et al. 2014), peanut (Özkan and Aasim, 2019; Day et al., 2017) and *Ginkgo biloba* (Isah, 2020) for the development of efficient and repeatable *in vitro* regeneration protocol.

The primary objective of *in vitro* regeneration experiments is to achieve high regeneration frequency. However, the application of PGRs in the culture

medium may lead to callus and regeneration induction simultaneously. Although callus induction was recorded on each explant, it was relatively more prominent on preconditioned explants compared to nonconditioned explants. However, it is dependent on other factors like the type of explant or genotype. Low calli induction due to preconditioning has been reported for dwarf chicklings (Sağlam, 2012) and peanut (Matand et al., 2013; Akasaka et al., 2000). The results of 100% regeneration frequency revealed no impact of preconditioning of peanut explants. These results are contrary to the previous findings of Matand et al. (2013) and Akasaka et al. (2000), who achieved low regeneration frequency from preconditioned explants of peanut. A recent study by Özkan and Aasim (2019) reported 100% regeneration of three different preconditioned explants of peanut. The results of this study suggested that the issue of low regeneration frequency of peanut reported in different studies (Venkatachalam and Kavipriya, 2012; Shan et al., 2009) can be overcome by adopting preconditioning technique. Similar results of high regeneration frequency due to preconditioning of explants have been reported for other crops also. Barpete et al. (2014) compared preconditioned embryonic node (2ip and TDZ) with nonconditioned explant of grass pea and achieved 100% shoot regeneration frequency from non-conditioned explants

Shoot counts followed by shoot length are other important parameters for the establishment of *in vitro* regeneration protocol. Results exhibited the superior impact of preconditioning of explants, which resulted in 2-fold more shoots. The more shoot counts from preconditioned explants might be due to rapid cell division by exposing explants to high cytokinin concentration (Aasim et al., 2013). The superior impact of preconditioning in shoot counts have been reported for other legume plants like lentil (Aasim 2012), chickpea (Aasim et al., 2013) and grass pea (Barpete et al., 2014). These results suggested that initial exposure of explants to high cytokinin concentration favours the multiple shoot induction. Day et al. (2017) achieved more shoot counts from preconditioned plumular apices with 20 mg/L BAP compared to plumular apices explants preconditioned with 10 mg/L BAP. Although initial preconditioning favours the shoot counts, it may exert a negative impact on shoot length. Previous studies revealed the mix impact of initial preconditioning on shoot length. Comparatively longer shoots in response to preconditioning have been reported for lentil (Aasim 2012) and chickpea (Aasim et al., 2013) compared to nonconditioned explants. Similar response of preconditioning with cytokinins on shoot regeneration has been reported for *Pongamia pinnata* (Belide et al., 2010) and *Sophora tonkinensis* (Jana et al., 2013). It is well documented that higher cytokinin concentrations in the culture medium suppress the shoot length. The possible reason for longer shoots might be due to the release of initial stress in the culture medium enriched by low cytokinin concentration.

Explants play a vital role and regulate the *in vitro* organogenesis under the control of other factors like presence of meristematic regions, PGRs type or concentration and growth conditions. The potential of explants can be enhanced by adopting variable techniques like treating explants with a higher concentration of cytokinins at the initial stage (Day et al., 2017; Barpete et al., 2014; Madhulatha et al., 2004). Results revealed the clear impact of explant type on shoot counts and shoot length. Each explant exhibited a different response, explants of ZE treatment were least responsive but induced shorter shoots. A study by Özkan and Aasim (2019) revealed the highest shoot counts and longer shoots from zygotic embryos, followed by plumular apices and embryonic axis explants. Whereas, Aasim et al. (2011) reported longer shoots from preconditioned embryonic explant compared to preconditioned zygotic embryo explants. These results suggest that pre-treatment type, dose and duration along with explant type regulate the shoot regeneration behaviour.

The combined effect of preconditioning of explants reveals the positive impact of initial preconditioning of explants with BAP on *in vitro* shoot regeneration of peanut. Results revealed 2-6 fold more shoot counts for preconditioned × explants for ZE and PA explants. These results confirmed the findings of Aasim (2012), who achieved 2-20 fold more shoots from preconditioned plumular apices explants compared to nonconditioned explants. Aasim et al. (2013) also achieved higher shoot counts from preconditioned plumular apices explants of chickpea compared to nonconditioned explants. Similarly, a positive impact of preconditioning of explants with 2ip and TDZ on shoot counts in grass-pea has been reported by Barpete et al. (2014). Recently, Özkan and Aasim (2019) investigated the preconditioned ZE, PA and EA explants of peanut and achieved highest shoot counts in order of ZE > EA > PA and ZE > PA > EE when cultured respectively on 1.0 mg/L BAP and 0.5 mg/L BAP.

The difference in the results might be due to more culture time in this study. Likewise, shoot counts, results on mean shoot length exhibited a significant impact and linkage between explant type and initial preconditioning. Preconditioning × explants generated longer shoots for ZE, PA and EA generated longer shoots for nonconditioned explants. These results suggested that both factors are responsible for generating longer shoots, and it can vary with explant or plant type. Earlier studies of our research group on lentil (Aasim, 2012) and chickpea (Aasim et al., 2013) revealed the longer shoots from preconditioned plumular explants compared to nonconditioned explants. Contrarily, Barpete et al. (2014) achieved longer shoots from nonconditioned embryonic node explants of chickpea compared to explants preconditioned with 10 and 20 mg/L of 2ip or TDZ.

Irrespective of preconditioning of explants, *in vitro* rooting, in legumes is considered difficult, including lentil (Aasim, 2012), chickpea (Aasim et al., 2013) and peanut (Hassan et al., 2013). Previous studies on *in vitro* regeneration of peanut from preconditioned explants revealed the high rooting frequency (Özkan and Aasim, 2019; Day et al., 2017; Day and Aasim 2017). A similar type of high rooting frequency was also achieved in this study by employing the protocol established by Özkan and Aasim (2019). Similarly, rooted plantlets were acclimatized successfully in the growth room followed the procedure established by Day et al. (2017) for different peanut cv (Halisbey) and Özkan and Aasim (2019) for the same cultivar (NC 7). The results on *in vitro* rooting and acclimatization reveal that preconditioning of explant does not have any negative impact and can be employed for other peanut cultivars.

5. Conclusion

Development of new *in vitro* regeneration protocol for recalcitrant plants for the application of biological techniques like genetic transformation is highly important. It is concluded that preconditioning of explants improves the regeneration frequency without showing any negative impact on subsequent rooting and acclimatization. However, preconditioning of explants exerted a negative impact on plant growth with stunted shoots and small leaf size. There is a need to optimize the proper concentration for preconditioning and exposure time to overcome this issue. This research work establishes a new vista in peanut biotechnology for facilitating the application of techniques like genetic transformation or *in vitro* screening against different abiotic stresses in order to improve peanut germplasm.

List of Abbreviations: 2iP, 2-isopentyl adenine; **BAP**, Benzylaminopurine; **IBA**, Indole-3-butyric acid; **MS**, Murashige and Skoog; **NAA**, Naphthalene Acetic acid; **NZE**, Nonconditioned zygotic embryos; **NPA**, Nonconditioned plumular apices; **NEA**, Nonconditioned embryonic axis; **PZE**, Preconditioned zygotic embryos; **PPA**, Preconditioned plumular apices; **PEA**, Preconditioned embryonic axis; **TDZ**, Thidiazuron.

Competing Interest Statement: Both authors declare that they have no competing interest.

Author's Contribution: Both authors have equal contribution to the planning, conduction and writing of the research article. Authors have read and approved the final manuscript. This work is the part of Master's thesis of Hülya Özkan under the supervision of Prof. Dr. Muhammad Aasim.

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