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Full Length Article

High-Frequency Direct Organogenesis from Cotyledonary Node Explants and Plantlet Regeneration of Peanut (*Arachis hypogaea*) Cultivars

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Abstract

The efficient plantlets regeneration of peanut is the most important and a primary step to successfully transform gene and apply recently developed genome editing techniques for crop yield improvement. The purpose of this research is to develop protocol for peanut cultivars applying different concentration of hormones for selected peanut cultivars and develop plantlets regeneration protocol. There is no previously reported protocol for the Chinese peanut cultivar N3 and Yu-hua-14. We optimized shoot and root regeneration protocol for two peanut cultivars. Both cultivars showed positive response for the cytokinin plant growth hormone 6-benzylaminopurine (BAP) and thidiazuron (TDZ). The highest shooting rate (97%) was found in a medium supplemented with 4 mg/L BAP and (94.33%) for 1 mg/L TDZ. Hence, more shoot initiation was observed at higher concentration of BAP as compared to TDZ. However, the maximum root regeneration (81%) was found on medium containing 0.3 mg/L 2, 4-dichlorophenoxyacetic acid (2,4-D) and the highest rooting rate (96.33%) was found in a medium containing 1 mg/L α-naphthalene acetic acid (NAA), indicating lower concentration of NAA induce more rooting rate than 2,4-D treatment. In this study, cotyledonary node was used, and this method was found to be efficient and rapid for *in vitro* peanut regeneration.

Keywords: Auxin; Cotyledonary nodes; Cytokinins; Peanut; Plant regeneration

Introduction

Cultivated peanut (*Arachis hypogaea* L.) is an important oil seed and grain legume crops of worldwide. Peanut is economically important legume, main source edible oil and source of food (El-Akhal *et al*. 2013; Meena *et al*. 2016) and its seeds are rich sources of dietary essential fatty acids including oleic and linoleic acids (Toomer 2017). The cultivated peanut has a genome size of 2.7 GB, is an allotetraploid (2n=4x=40, AABB) plant species derived from two diploid wild peanut species, (*Arachis duranensis* (A genome) and *Arachis ipaensis* (B genome) (Grabiele *et al*. 2012; Moretzsohn *et al*. 2013; Bertioli *et al*. 2016). The average production share of peanut with shell by region from 1994–2019 were 64, 8.6, 27.3, 0.1 and 0% in Asia, America, Africa, Oceania and Europe respectively. China and India are the largest peanut producer in the world with the total production of 17,519, 600 and 6,727,180 tonnes per year respectively (FAOSTAT 2019).

Peanut is considered recalcitrant to tissue culture and methods such as *in vitro* propagation helps for mass propagation. (Heatley and Smith 1996; Akasaka *et al*. 2000), efficient and successful protocols using different explant sources were developed. Several *in vitro* regeneration using different explant sources have been reported in peanut, including: epicotyl (Little *et al*. 2000; Shan *et al*. 2009), immature leaflet (Venkatachalam *et al*. 1999; Tiwari and Tuli 2009), hypocotyl (Venkatachalam *et al*. 1997; Matand and Prakash 2007), somatic embryos (Hazra *et al*. 1989; Joshi *et al*. 2003), cotyledonary node (Banerjee *et al*. 2007; Hsieh *et al*. 2017; Limbua *et al*. 2019), cotyledon (Baker and Wetzstein 1995; Masanga *et al*. 2013), seed (McKently *et al*. 1990) and leaf segment (Akasaka *et al*. 2000). In all the regeneration frequency varied due to explant sources and the type and concentration of hormone used. Kenyan peanut genotypes, ICGV12991, CG7 and Red Valencia have been successfully regenerated using cotyledonary node and reported a regeneration frequency of 80 to 81% (Limbua *et al*. 2019). On the other hand, 86 and 98% were reported shooting rate with no significance difference at different concentration of BAP (Hsieh *et al*. 2017). Also legume crops regenerated in *in vitro* culture using different explant sources

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have been reported in soybean including: immature embryonic axes and cotyledonary node (Pathak *et al*. 2017), chickpea plumular apices (Aasim *et al*. 2013), pigeon pea cotyledonary node and mungbean cotyledonary node (Mojumder *et al*. 2015).

In addition to regenerating shoot buds and/or developing roots using different explant sources through tissue culture methods, are also very important in the development of transgenic plants. For instance, to transform gene using Agrobacterium mediated gene transformation, efficient plant regeneration method and appropriate explant are useful in crop breeding programs. The most important and primary step for transfer gene efficiently to plant species is the presence of appropriate protocol for genetic transformation that is well-suited with *in vitro* plant regeneration technique of the selected and targeted plant species (Kar *et al*. 1996). Some scientific research findings have been reported in peanut (Sharma and Anjaiah 2000; Anuradha *et al*. 2006; Bhatnagar *et al*. 2010). These researchers reported that using CNs for *in vitro* regeneration of various plant species confirmed that it is best mechanism for the development and production of enormous number of independently transformed plants. Similarly, Hsieh *et al.* (2017) reported that direct regeneration by using CN reduce time in tissue culture system to develop healthy and reproducible plants and it is suitable for genetic transformation.

In our study we used two Chinese peanut cultivars for protocol optimization by applying different concentrations of hormones. There is no previously developed protocol for the peanut cultivars studied. Hence the main goal of the present paper was to establish plant regeneration system from cotyledonary node (CN) and to evaluate suitable plant growth hormone concentration for the Chinese peanut cultivar.

Materials and Methods

Explant Preparation and *in vitro* Culture Condition

Mature and healthy seeds of peanut cultivar Yu-hua-14 and N3 were used which was previously stored stock in the department of crop genetics and breeding, Jilin Agricultural University, China. The embryo axes were removed from the dry seed and soaked for about 14 h in sterilized double distilled water. Surface disinfected in 10% (w/v) NaOCl solution for 7 and 1 min in 70% (w/v) ethanol and washed three times in sterilized distilled water for 6–7 min each. The embryo axes were germinated in glass jar. MS salts with vitamin and sucrose were purchased from Coolaber Science and Technology Co., Ltd, Beijing, China. 6-benzylaminopurine (BAP), TDZ and agar were purchased from Shanghai Aladdin Biochemical technology Co., Ltd, China. 0.8% (w/v) agar and 3% (w/v) sucrose were used in 1L growth medium preparation. pH was adjusted at 5.7 before autoclaving. The plantlets growth conditions were 25/25°C day/night, 16h photoperiod and 130–150 μmol m^{-2} s^{-1} florescent light. After three weeks cotyledonary nodes were removed as described (Hsieh *et al.* 2017). Elongated shoots were placed on to root induction medium (RIM).

Optimization of Shoot Induction and Elongation Media

To evaluate the effect of TDZ, 2 mg/L were used in the shoot induction media (SIM) and the cotyledonary nodes were transferred to SEM with 0, 0.5, 1, 2 mg/L TDZ. Different concentration of BAP (0, 1, 2, 3, 4, 5 mg/L) were applied in both SIM and SEM. The experimental design was completely randomized block design with three replicates and each jar contained ten CNs. After 1month of shoot development half of the CNs were used to measure phenotypic data (shoot length and fresh shoot weight).

Optimization of Root Induction Media

Two different auxins, 2, 4-D and NAA were used separately in RIM. Shoots initially grown in 4 mg/L BAP SIM and SEM, 2 mg/L TDZ SIM and 1 mg/L TDZ SEM were transferred to RIM with different concentrations of 2, 4-D (0, 0.1, 0.2, 0.3 mg/L) and NAA (0, 1, 2, 3, 4 mg/L). The experimental design was completely randomized block design with three replicates and each glass jar contained five shoots. After one month of root initiation, phenotypic data such as rooting rate, root number, root fresh weight and length were recorded.

Statistical Analysis

Each experiment had a completely randomized block design with three replicates. Standard deviation and means separations were calculated according to Takey‘s Multiple Range Test. All statistical analysis were performed using Minitab17 software (Minitab Inc., State College, PA, USA). Analysis of variance (ANOVA) was used to test statistically significant difference between cultivars.

Results

Effect of BAP on Shoot Induction

Mean value for shooting rate ranged from 84–97% for the peanut culttivar N3 wheras for Yu-hua-14 it ranged from 85–95%. The maximum shooting rate (97%) for N3 and 95% for Yu-hua-14 obtained in a medium containing 4 and 5 mg/L BAP, respectively (Fig. 1b) and shoot length was decreased as the concentration of hormones increased, but it is significantly decreased at 2 mg/LBAP. Average number of shoot length (6.66) and (6.33) was found in N3 and Yu-hua-14 at 4 mg/L BAP concentration (Fig. 1d). The shoot number ranged from 1 to 4.33 for N3 and 1.33 to 4.66 for Yu-hua-14 (Fig. 1c). The maximum shoot number 4.66 and 4.33 was obtained on MS medium containing 5 mg/L BAP for Yu-hua-14 and N3 respectively (Fig. 1c). Suggesting that, the shoot

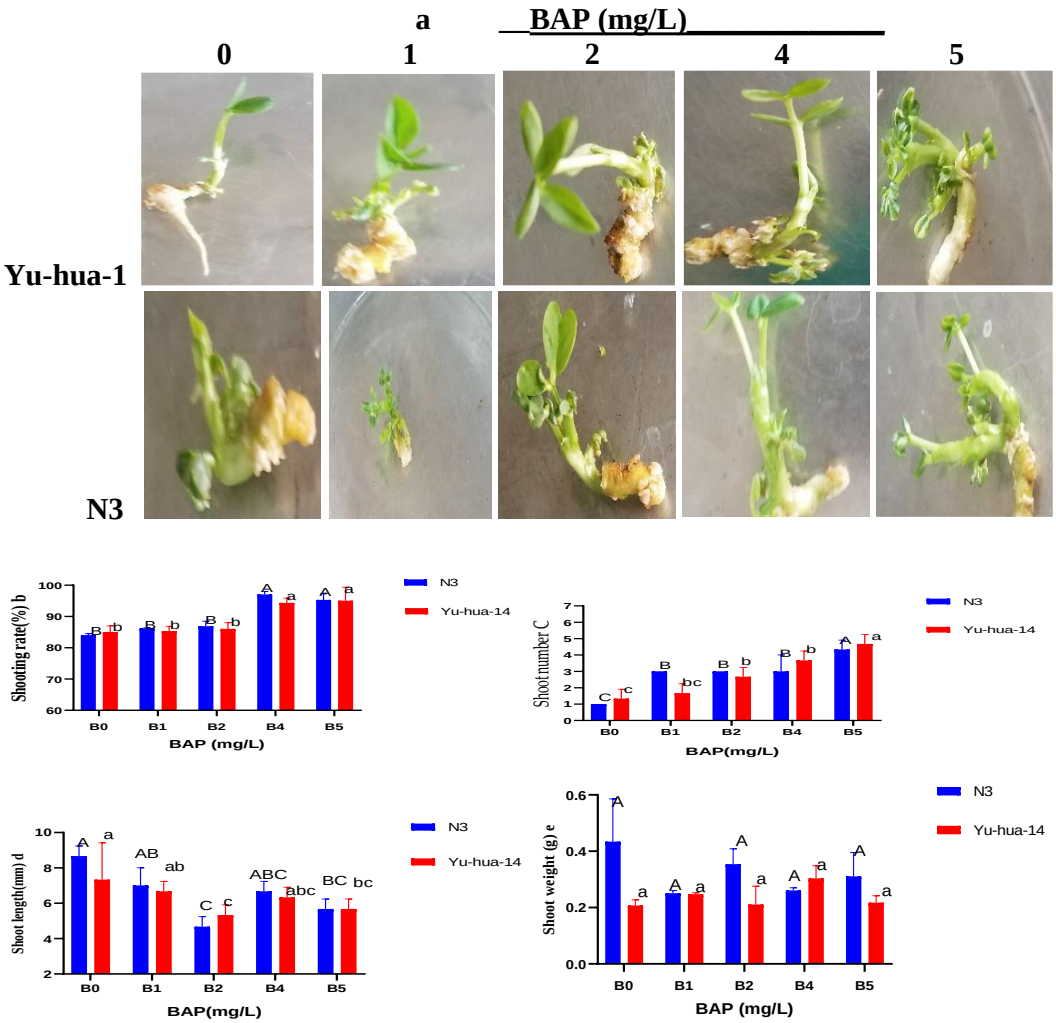

Fig. 1: 6-benzylaminopurine effect on peanut cultivar Yu-hua-14 and N3. a three-week-old Yu-hua-14 and N3 peanut cultivar under different concentration of BAP treatment, b shooting rate, c shoot number, d shoot length, e shoot weight

number increased as BAP hormone concentration increased. On the other hand, shoot length was found medium as BAP treatment increased for both cultivars (Fig. 1d). There was no significance difference recorded in shoot weight between cultivars (Fig. 1e). From the result we conclude that 4 mg/L BAP was prefereble for both cultivars to produce healthy shoot number and shoot length. There were significant difference in shoot number ($p < 0.001$) and shoot length ($p< 0.001$) for both cultivars at different concentration of BAP (Fig. 1c, d).

Effect of TDZ on Shoot Induction

To evaluate and optimize the influence of TDZ hormone on shoot initiation and elongation of peanut cultivars we used different concentration of TDZ (0, 0.5, 1, 2 mg/L). Among the four phenotypic parameters we recorded that shooting rate (Fig. 2b), shoot number (Fig. 2c), shoot length (Fig. 2d) and shoot weight (Fig. 2e). We recorded that shooting rate ranged from (80.33–94.33%) for N3 and (81.33–94%) for Yu-hua-14 (Fig. 2b). The number of shoots was considerably increased from (1.67–5.00) for N3 and the maximum shoot number was found in 2 mg/L TDZ while medium shoot number was obtained 4.33 and 3.66 for the cultivar N3 and Yu-hua-14 in a medium containing 1 mg/L TDZ. Shoot number ranged from (2–5), (2.67–5.00) in a medium containing 0.5 mg/L TDZ for N3 and Yu-hua-14 respectively (Fig. 2c). We observed morphologically thin shoots under TDZ treatment on SEM and it caused some morphological variation in shoots (Fig. 3a, b). There was significant difference in shoot number ($p< 0.001$) and shoot length ($p< 0.001$) for both cultivars at different level of TDZ treatment (Fig. 2c, d).

Maximum number of shoots (5) was recorded at 2 mg/L TDZ concentration for both cultivars (Fig. 2c). The study showed that TDZ was effective in forming shoots in peanut cultivar. The average shoot length (mm) decreased from (8.00–4.67) for N3 and (7.00–4.67) for Yu-hua-14, indicating

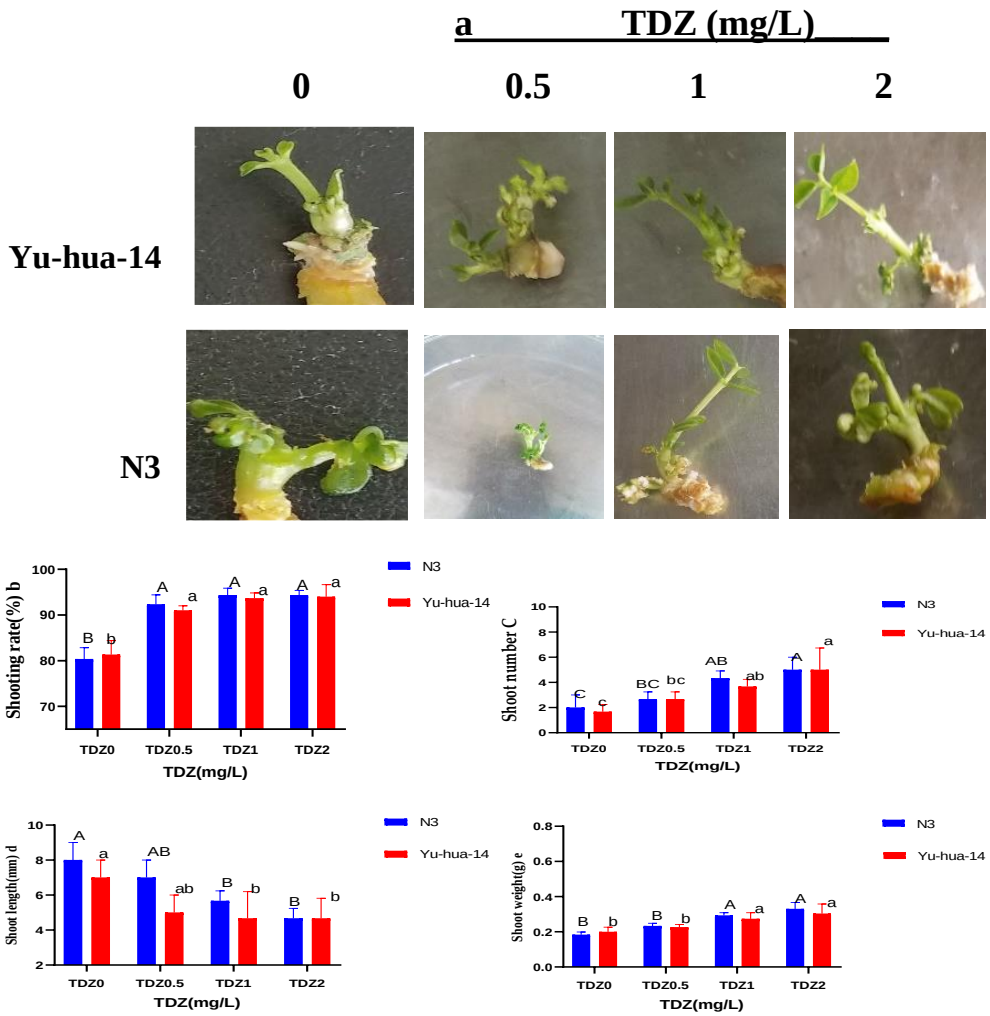

Fig. 2: Thidiazuron effect on peanut cultivar Yu-hua-14 and N3 at different concentration of TDZ. a three-week-old Yu-hua-14 and N3 peanut cultivar under different concentration of TDZ, b shooting rate, c shoot number, d shoot length, e shoot weight

the shoot length decreased as the concentration of TDZ increased (Fig. 2d). Maximum shoot weight (0.33) was found at the 2 mg/L TDZ for N3 cultivar. There was no significance difference observed in a medium containing TDZ treatment for shoot weight.

Effect of 2,4-D and NAA on root regeneration: Initially grown at BAP medium

Responses of CNs cultures to different concentration of 2, 4-D and NAA on RIM are shown in Fig. 4, 5 that includes the development of roots. The development of roots was observed after 7 days of growth on RIM. The root induction increased with an increase in 2, 4-D. At 0.3 mg/L 2, 4-D of the medium, highest (80.67%) rooting was found for Yu-hua-14 and (80.33%) for N3 (Fig. 4b).

Root number and root fresh weight increased as the concentration of 2, 4-D increased. However, the level of 2, 4-D exceeds 0.2 mg/L, root number decreased (Fig. 4c. e). Further increments in 2, 4-D level did not improve number of root formation in peanut. Root length was obtained high at zero concentration (Fig. 4d). There was no significance difference at 0.1, 0.2 and 0.3 mg/L 2, 4-D concentration for both cultivars.

The highest rooting rate (96.33%) and root number (10) for N3 cultivar were obtained on RIM containing 1 mg/L NAA and 4 mg/L NAA respectively (Fig. 5b, c). Root induction rate was decreased as the level of hormone exceeded 1 mg/L NAA for N3. However, for the cultivar Yu-hua-14, root induction decreased as level of hormone exceed 2 mg/L NAA (Fig. 5b). Root length was decreased as the concentration of auxin increased. Lower hormone concentration is required to induce root. The root fresh weight ranged between 0.233 to 0.57 for Yu-hua-14 and 0.17 to 0.566 for N3. The maximum root fresh weight (0.57) was found in a medium supplemented with 4 mg/L NAA for Yu-hua-14 (Fig. 5e). Both cultivars showed positive response for NAA and the roots first initiated at 7th day of culture.

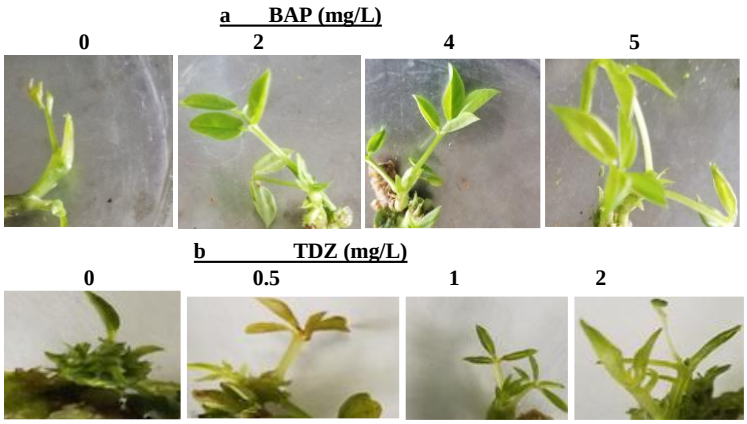

Fig. 3: Effect of BAP or TDZ on the peanut Yu-hua-14 cultivar for 1month.a shoot from CNS with BAP treatment b shoot from CNS with TDZ treatment

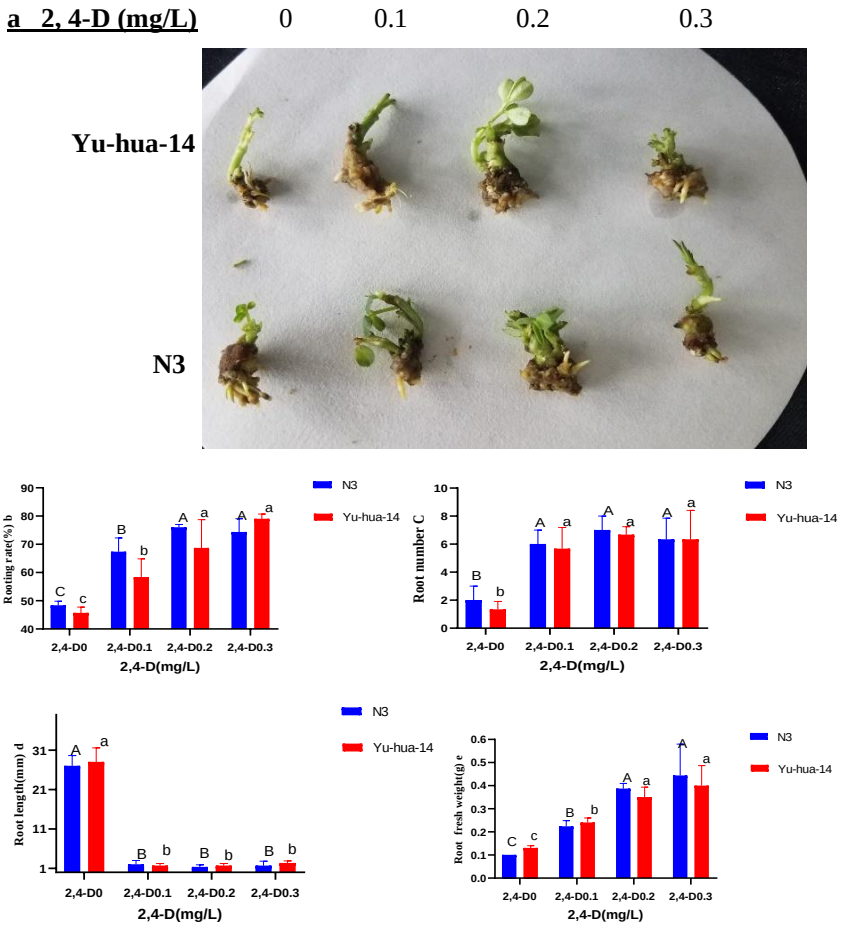

Fig. 4: Effect of 2, 4-D on the in vitro response of peanut cultivar Yu-hua-14 and N3 at different concentration of auxin treatment initially grown on BAP medium. a 1-month-old Yu-hua-14 and N3 peanut cultivar under different concentration of 2,4-D treatment, b rooting rate, c root number, d root length, e root fresh weight

Effect of 2, 4-D and NAA on Root Regeneration: Initially Grown at TDZ Medium

For root regeneration, peanut cultivars initially grown on TDZ medium was tested at different concentration of 2, 4-D and NAA. Regenerated roots are shown (Fig. 6a, 7a). The root regeneration we observed in 2,4-D treatment initially grown at TDZ medium was less effective. The maximum rooting rate (81%) was found for Yu-hua-14 in a medium supplemented with 0.3 mg/L 2, 4-D (Fig. 6b), indicating 2, 4-D

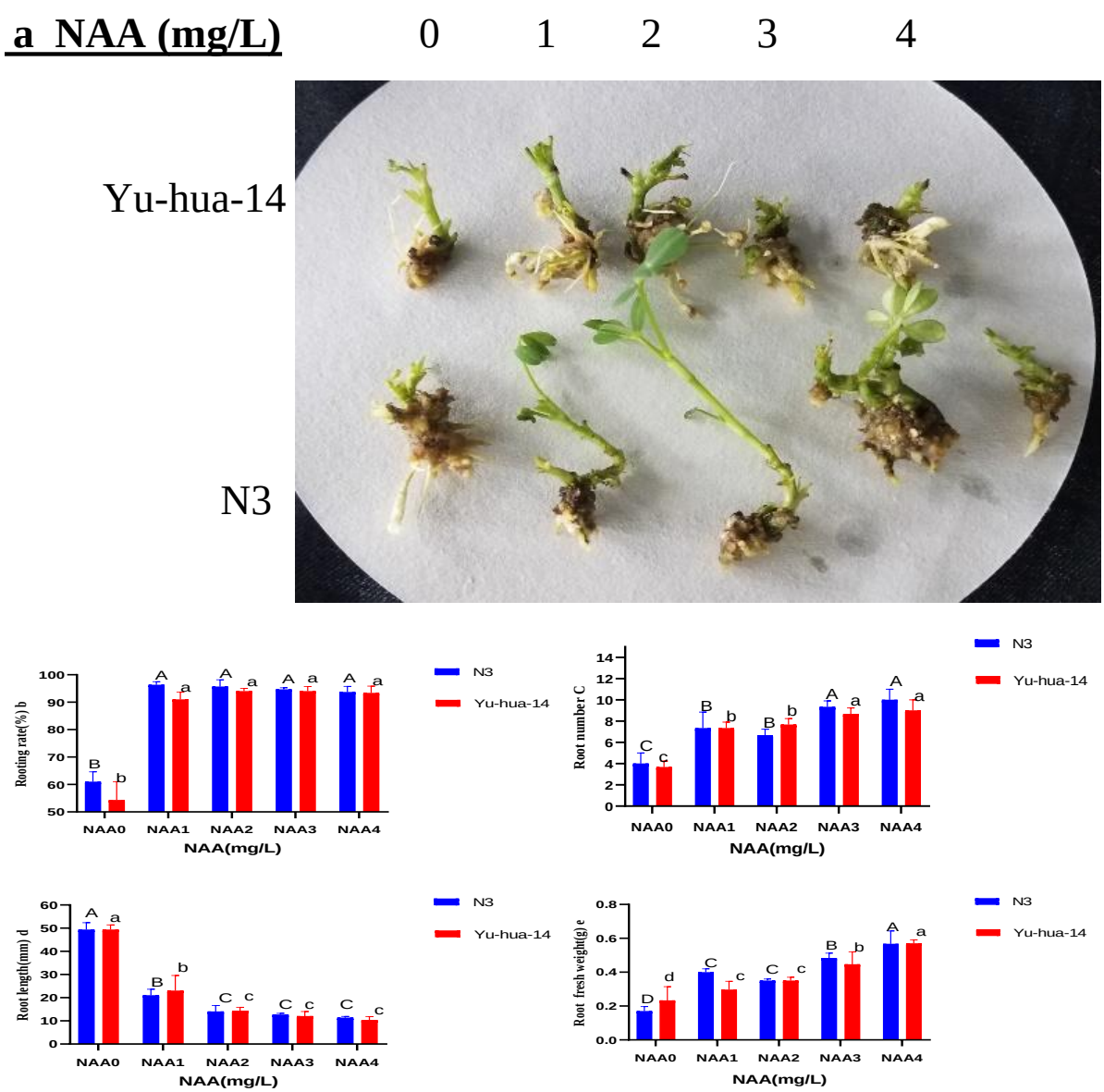

Fig. 5: Effect of NAA on the in vitro response of peanut cultivar Yu-hua-14 and N3 in different concentration of auxin treatment initially grown on BAP medium. a 1-month-old Yu-hua-14 and N3 peanut cultivar under different concentration of NAA treatment, b rooting rate, c root number, d root length, e root fresh weight

was not effective hormone for regeneration of roots as compared to NAA (Fig. 6b, 7b). Root number and root fresh weight increased somewhat as the concentration of hormone increased (Fig. 6c, e).

It was observed that both cultivars regenerated maximum rooting rate (94%) (Fig. 7b). The highest root number 10 was found at 4 mg/L NAA and root length decreased as the concentration of NAA increased for both cultivars (Fig. 7c, d). Root fresh weight ranged from 0.1–0.56 g for N3 and 0.14–0.52 g for Yu-hua-14 (Fig. 7e). The result shows there was TDZ influence on the root formation, therefore, further investigation is needed.

Discussion

For peanut plantlet regeneration and development of healthy plants an efficient regeneration system is an important and basic step for applying genetic transformation in the plant species. Several research findings had reported for peanut using different explant sources. Some reports had shown low regeneration frequency (34.7%) for the generation of plantlets (Akasaka *et al.* 2000) and takes long time *i.e.* about 4 months (Tiwari and Tuli 2009; Akasaka *et al.* 2000). However, few reports showed that using cotyledonary node is effective and time efficient (Hsieh *et al.* 2017).

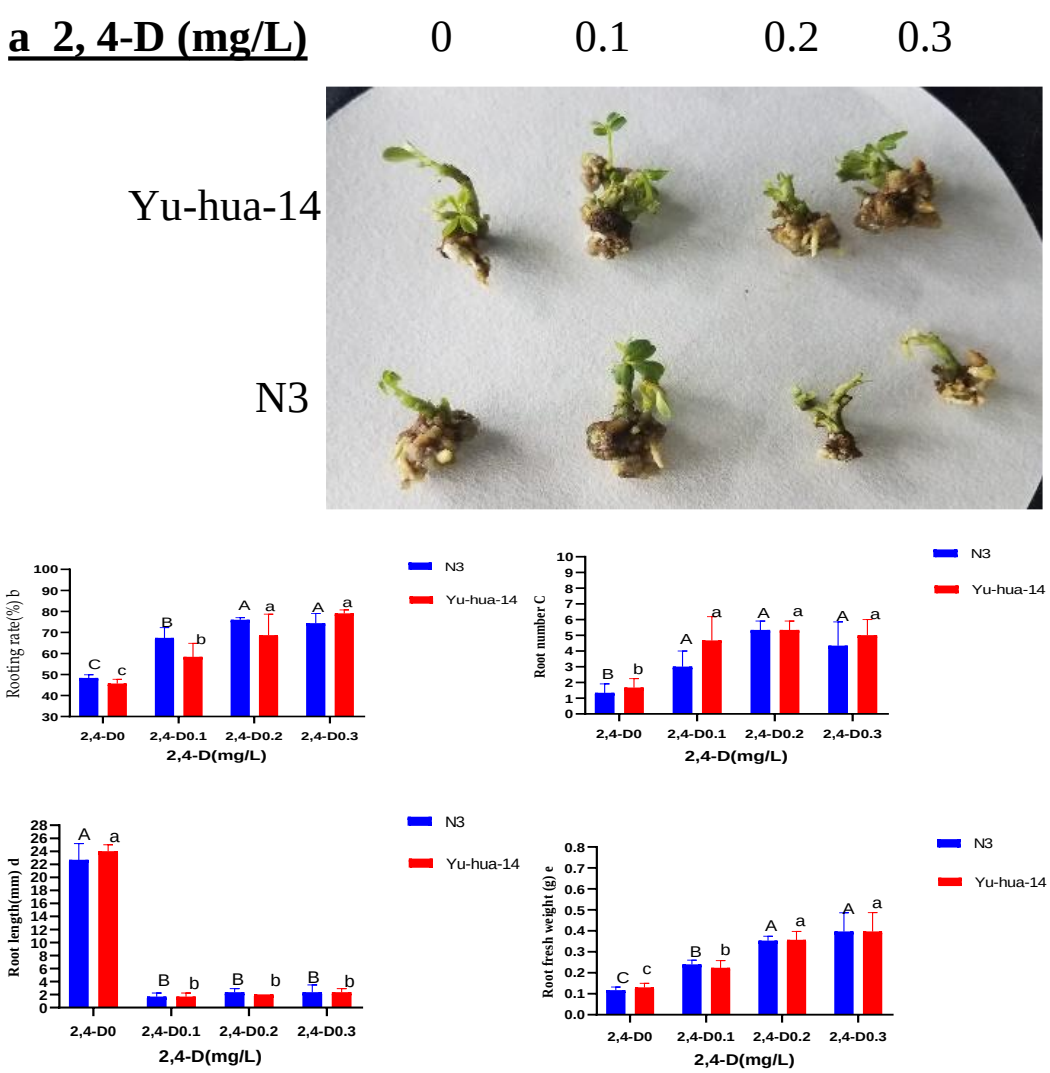

Fig. 6: Effect of 2, 4-D on the in vitro response of peanut cultivar Yu-hua-14 and N3 in different concentration of auxin treatment initially grown on TDZ medium. a 1-month-old Yu-hua-14 and N3 peanut cultivar under different concentration of 2,4-D treatment, b rooting rate, c root number, d root length, e root fresh weight

The effect of different concentration of TDZ and BAP were tested in order to develop peanut through *in vitro* regeneration (Fig. 1 and 2). *In vitro* regeneration of peanut is difficult because of its recalcitrant nature. However, we successfully developed protocol from cotyledonary node. The explants developed regenerant shoot buds from CNs within 3 weeks of culture. BAP hormone concentration (1–5 mg/L) tested (Fig. 1b) and TDZ concentration (0.5–2 mg/L) generated shoots (Fig. 2c). Some reports witness that MS medium containing different combinations of hormones regenerated maximum number of shoot buds. For instance, Limbua *et al.* (2019) reported (98%) in 5 mg/L BAP and 1 mg/L TDZ. Tiwari and Tuli (2009) and Palanivel *et al.* (2002) reported (77.76–81.5%). However, using BAP alone, we found highest shooting rate (97%). The Percentage of shoot regeneration differed across various BAP treatment. From the two cultivars evaluated, N3 responded best with the highest shooting rate of (97%) on medium containing 4 mg/L BAP and (94.33%) on 1 mg/L TDZ. Hence, higher shoots were observed at BAP treatment than TDZ. This suggested that BAP is an effective growth regulator for peanut shoot regeneration. Previous studies on lentil (Chhabra *et al.* 2008), peanut (Gill and Saxena 1992) and soybean (Kaneda *et al.* 1977) reported that lower concentration of TDZ than BAP were effective for shoot organogenesis. The present report indicated that both cultivars (Yu-hua-14 and N3) responded positively for both BAP and TDZ and shoot were regenerated from the cotyledonary nodes between the two peanut cultivars, indicating that shoot regeneration using CNs might be cultivar independent (Sanyal *et al.* 2003; Hsieh *et al.* 2017; Limbua *et al.* 2019).

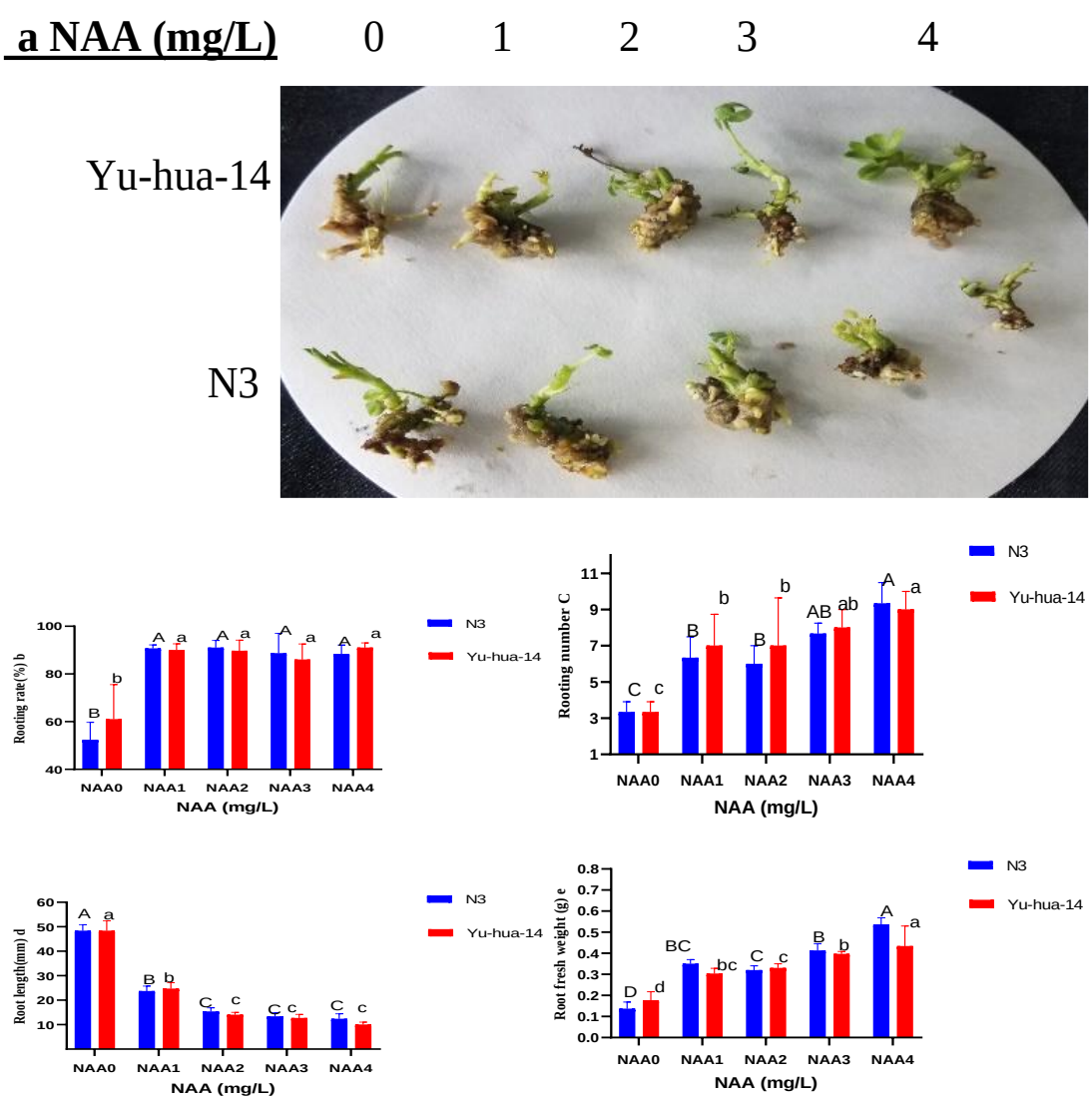

Fig. 7: Effect of NAA on the in vitro response of peanut cultivar Yu-hua-14 and N3 in different concentration of auxin treatment initially grown on TDZ medium. a 1-month-old Yu-hua-14 and N3 peanut cultivar under different concentration of NAA treatment, b rooting rate, c root number, d root length, e root fresh weight

To evaluate and optimize the effect of 2, 4-D and NAA on two peanut cultivars we applied two hormones separately in RIM for both cultivars. Visual observations were made periodically after 6 days of culture on RIM. Root development was initiated at seventh day on a medium containing NAA. Healthy roots were formed after one month of root induction and all rooted plantlets grew normally (Fig. 8a–f). The maximum root regeneration (81%) was found on medium containing 0.3 mg/L 2, 4-D for the peanut cultivar Yu-hua-14 which was grown initially at TDZ medium (Fig. 6b). However, the maximum rooting rate (80.67%) was found on medium containing 0.3 mg/L 2, 4-D initially grown on BAP medium (Fig. 4b). On the contrary, a highest rooting rate (96.33%) was noted in a medium containing 1 mg/L NAA for the peanut cultivar N3 which was initially grown at 4 mg/L BAP medium (Fig. 5b). On the other hand, high rooting rate (94%) was obtained on Murashige and Skoog (MS) medium containing 2 and 3 mg/L NAA, which were initially grown on TDZ shoot initiation and elongation medium (Fig. 7b) (Murashige and Skoog 1962). From this study we can understand that treatment with lower concentration of NAA *in vitro* culture system would be preferable to produce and develop phenotypically healthy peanut. We found NAA to be better than 2,4-D in developing roots. This is in agreement with Hsieh *et al*. (2017). In the present study we observed that both TDZ and BAP has effect on morphological features of peanut cultivars (Fig. 4–7).

Some previous reports indicated that root regeneration varied among different concentration of hormone treatment.

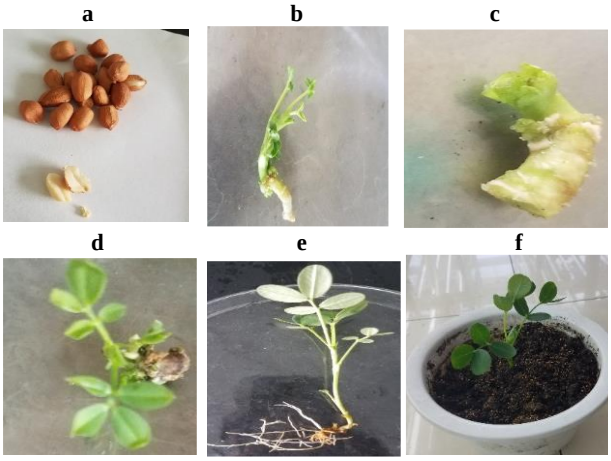

Fig. 8: Plantlet's regeneration system of peanut variety Yu-hua-14. a mature seeds and embryo, b 3-week-old regenerated peanut plantlets, c cotyledonary node, d 3-week-old shoots regeneration, e regeneration of roots, f regenerated plants transferred to soil

A maximum frequency of root regeneration (68.3%) on MS medium supplemented with 2 mg/L BAP and 1.5 mg/L NAA in black gram (Adlinge *et al*. 2014), 93.3% on MS medium with 1 mg/L NAA in peanut (Masanga *et al*. 2013) and 100% on medium containing 5.7 µM NAA in peanut (Hsieh *et al*. 2017) were reported. In the present study the percentage of rooting success increased at lower auxin concentration. This finding disagrees with (Banerjee *et al*. 1988; Palanivel *et al*. 2002).

Phenotypic data for root were recorded for both cultivars that were initially grown at 4 mg/L BAP medium. Accordingly, a maximum root number (7 ± 0.577) was recorded at 0.2 mg/L 2, 4-D treatment (Fig. 4c) and maximum root number (10 ± 0.577) was obtained at 4 mg/L NAA. However, root length was the longest on RIM with zero 2, 4-D and NAA (Fig. 4d, 5d). Root fresh weight was maximum (0.443) on RIM with 0.3 mg/L 2, 4-D for Yu-hua-14 and (0.57) on RIM with 4 mg/L NAA for N3 cultivar (Fig. 4, 5). Additionally, to evaluate the effect of 2,4-D and NAA, we measured root morphological data for peanuts that were initially grown at 2 mg/L TDZ shoot initiation and 1 mg/L TDZ shoot elongation medium. A maximum root number (10) was obtained on a medium containing 4 mg/L NAA for both cultivars, while at 0.2 and 0.3 mg/L 2,4-D the highest root number (6) was obtained (Fig. 6, 7). In this case root number increased as the concentration of hormone increased. In all auxin treatments, root length were highly decreased as compared to zero hormone treatment and the longest root were recorded at zero 2,4-D and NAA medium (Fig. 5–7).

Conclusion

Of the cultivars evaluated, N3 responded better with the highest shooting rate of (97%) on medium containing 4 mg/L BAP and (94.33%) on 1 mg/L TDZ. Hence, shoots were initiated more efficiently at higher concentration of BAP than TDZ, suggesting BAP was an effective growth regulator for peanut shoot regeneration. On the other hand, the highest rooting rate (96.33%) was obtained in a medium containing 1 mg/L NAA for the peanut cultivar N3, which indicated lower NAA level in *in vitro* regeneration system would be superior to produce phenotypically normal peanut plants and NAA was better than 2,4-D in initiating and producing roots.

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Author Contributions

AL conducted experiment, wrote and revised the manuscript. JZ, SY, XH supervised the whole process and revised and edited the manuscript. AL, AAM, DY, XL, MRC, Q W, JP H, YX & BS analyzed data. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare there is no conflicts of interest.

Data Availability

Data included in this paper will be available on a fair request to the corresponding author.

Ethical Approval

Not applicable in this paper.

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