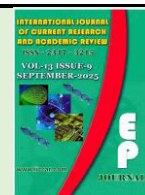




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Indirect *in Vitro* Regeneration of *Plectranthus edulis* From Shoot Tip and Internode Explants

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Abstract

Plectranthus edulis is a highly important tuber crop due to its medicinal and nutritional value. However, its production has been threatened due to the insufficiency of good propagation material. Therefore, a study was conducted to develop a protocol for the Indirect *in vitro* regeneration of *P. edulis* from shoot tip and internode explants. The results revealed that surface sterilization with 0.5 and 1% NaOCl for 10 minutes was effective for disinfecting shoot tip and internode explants, respectively. In callus induction, the highest callus induction percentages (100% and 93.75±6.25%) were obtained from 1.5 and 2mg/l 2, 4-D that supplemented MS media for shoot tip and internode explants, respectively. The highest percentage of shoot regeneration was achieved (66.67±8.33%) for shoot tip and internode explants cultured on MS medium with 0.8 and 1mg/l BAP. The highest multiplication rate (9.89) in the multiplication stage was obtained from MS medium supplemented with 1.5mg/l BAP. High rate (100%) root induction and root number (9.34) were achieved on half MS medium supplemented with 1.5mg/l and 2mg/l IBA, respectively. Acclimatization of plantlets with river sand and forest soil with 1:2 ratios gave 57.14% survival for both explants. The results of the study demonstrated a successful protocol for micro-propagation of *P. edulis* (212464/1984) through indirect organogenesis that can be used to address its production constraints. To determine its reproducibility and further enhance the efficiency of laboratory-based propagation protocol, it is essential to incorporate additional accessions and various combinations of PGRs.

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Introduction

Plectranthus edulis (Vatke) Agnew is a herbaceous dicotyledonous plant that belongs to the family Lamiaceae (Mekbib and Weibull, 2012). The genus *Plectranthus* has about 30 wild species in Ethiopia (Zerihun, 2022). *P. edulis* is the indigenous and underutilized tuber crop species in Ethiopia (Feyissa, 2021; Zerihun, 2022), which is commonly produced by

small-scale farmers following traditional practices and is highly localized in specific places and communities in the country (Mitiku and Roro, 2020; Abdena and Bedassa, 2023).

It is mainly cultivated in the south and southwestern parts of the country at altitudes ranging between 1880 and 2200 m a.s.l as food and sometimes medicine (Mekbib and Weibull, 2012; Fanta *et al.*, 2018).

P.edulis is grown mainly for its tubers for domestic consumption and rarely for commercial purposes (Mitiku and Roro, 2020; Feyissa, 2021; Fekadu *et al.*, 2022). This crop is rich in major nutrients (carbohydrates) and minor nutrients (vitamins and minerals) and is comparable to important root and tuber crops such as potatoes and sweet potatoes (Mekbib and Weibull, 2012; Melaku and Duguma, 2016). However, current production is declining and is even being lost completely in some areas. The main factors contributing to the decline in production include lack of planting material, poor tuber-keeping quality and long time to maturity, lack of knowledge of diseases, lack of varieties and inadequate agricultural practices (Feyissa, 2021). Therefore, tissue culture technology is an effective way to a rapid propagation system, conservation and improvement of this crop (Fanta *et al.*, 2018; Aschale and Feyissa, 2019). However, there were barriers in tissue culture systems, together with the availability and quality of plant materials for culture initiation; thus, various parts of a plant, such as shoot tip and internode, could be used as better explant sources with greater potential due to their abundance.

So far, several studies have been conducted on *in vitro* protocols for meristem culture (Tsegaw and Feyissa, 2014), shoot tip and nodal cultures (Kebede and Abera, 2014), somatic embryogenesis from leaf-derived callus (Fanta *et al.*, 2018) and organogenesis from *in vitro* leaf derived callus (Aschale and Feyissa, 2019) of *P.edulis*. However, there is lack of indirect organogenesis protocols for this specie. Different explants also have different regeneration abilities when treated with various PGRs and concentrations (Alexandrov *et al.*, 2021). So, the current study was aimed at optimizing indirect *in vitro* regeneration of *P.edulis* from shoot tip and internode explants by using different auxin and cytokinin hormone combinations and concentrations to boost *P.edulis* propagation in this investigation. Further, this study will serve as a support system for other future biotechnological advances like synthetic seed production, cryopreservation and secondary metabolite production while being a valuable tool in conservation.

Materials and Methods

Description of the Study Area

The study was conducted at Hawassa University, College of Agriculture, School of Plant and Horticultural Sciences in Plant Cell and Tissue Culture Laboratory during the period from January 2023 to February 2024.

Located at latitude of 7°04'N and a longitude of 38°31'E, the area sits at an altitude of 1,700 meters above sea level, with average annual rainfall ranging from 900 to 1,100 mm. The minimum and maximum temperatures throughout the year are 12°C and 27°C, respectively.

Planting material

P.edulis germplasm (Accession ID, 212464/1984) was collected from the Ethiopian Biodiversity Institute (EBI), Choche field gene bank. The tubers of *P.edulis* were cut into pieces and planted in prepared soil mixtures consisting of forest topsoil and compost in a ratio of 2:1, respectively, on the plastic pots. Tubers were planted in a shade house at 25±2°C, 60% relative humidity, and a 12-h photoperiod in Hawassa University College of Agriculture for donor plant establishment. They were watered every four days from the planting date using a spray can. Weeding and earthing up were performed as needed to enhance aeration and control infections. After one month of growth, healthy and vigorous young shoot tips and internode segments were collected to serve as explants. The top part of the auxiliary and apical shoot tips and the first 2-3 internodes from the top of the plantlet were excised for internode explants. The accession was chosen because of its potential for high yield, grower's interest, and it corresponds to the main cultivated material in the area.

Culture media preparation

The culture medium was prepared using the appropriate amounts of Murashige and Skoog (1962) stock solutions (mg/l) sourced from Sigma-Aldrich, St. Louis, MO, USA. For callus induction, shoot initiation, and multiplication, full-strength MS medium was utilized, supplemented with 30 g/l of sucrose as a carbohydrate source (w/v). In contrast, half-strength MS medium with 15 g/l of sucrose was employed for root induction. The pH of the medium was adjusted to 5.8 using 1N NaOH and 1N HCl after the addition of growth regulators. Agar (8 g L⁻¹; Phytotechnology™, USA) was incorporated as a gelling agent. The media were then sterilized by autoclaving at 121°C under a pressure of 15 PSI for 15 minutes and subsequently stored at room temperature.

Plant growth regulators stock solutions

The Plant Growth regulators (PGRs) used for the study were: the cytokinin, 6-benzyl aminopurine (BAP), the auxin 2,4-Dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA) and α -naphthalene acetic acid (NAA).

All PGR stock solutions were prepared by weighing and dissolving the powder in distilled water at the ratio of 1mg/ml. To begin the dissolving process, the powdered crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1N NaOH and 1N HCl based on the type of PGR (NaOH for auxins and HCl for cytokinin) (Zamir *et al.*, 2012). The volume was then adjusted by the addition of distilled water. Ultimately, stock solutions of the growth regulators were prepared, labeled with the name of the solution, preparation date and the name of the person who prepared the solution and stored in a refrigerator at 4°C.

Explants and surface disinfection

For surface sterilization, shoot tip and internode explants were thoroughly washed under running tap water for 15 minutes to remove the surface contamination. And under a laminar airflow hood, explants were washed with two drops of Tween 20 and rinsed until clear with distilled water. Then, the explants were dipped in mancozeb (2g/l) for 15 minutes and then rinsed three times with autoclaved distilled water. Beneath a sterile laminar airflow hood, the explants were treated with 70% (w/v) ethanol for 45 seconds, followed by four rinses with autoclaved distilled water. Subsequently, the explant materials underwent surface sterilization using two different concentrations of local bleach (sodium hypochlorite from Ethiopia), specifically 0.5% and 1% active chlorine, for exposure times of 5, 10, and 15 minutes. (Fanta *et al.*, 2018) with modification. After that, the explants were rinsed with autoclaved double distilled water five times to remove the residual effect of this sterilant. Once the sterilization process was finalized, individual shoot tip and internode explants were aseptically trimmed into segments of approximately 1 cm². These segments were then positioned horizontally in contact with the MS basal medium. The effects of sodium hypochlorite concentration and exposure duration were evaluated for both types of explants cultured on PGR-free media at two-week intervals.

Callus induction

Surface-sterilized shoot tip and internode explants were cut into approximately 1 cm² segments and placed in sterile petri dishes (90 cm) containing full-strength MS basal medium. This medium was supplemented with various concentrations of BAP (0, 0.5, 1, 1.5 mg/l) and NAA (0, 1, 1.5, 2 mg/l) in combination, as well as 2,4-D alone at concentrations of (0, 1, 1.5, 2 mg/l) to assess callus induction. Each petridish contains four explants

and each treatment was replicated three times. The induced calli were subcultured to a fresh medium of the same composition after a month for the callus maturation for three weeks. All cultures were incubated at 25 ± 2°C in darkness to facilitate callus formation and prevent greening of the callus. The percentage of callus induction, morphological characteristics of the callus, callus diameter, and fresh weight of the callus were assessed at four-week intervals following the inoculation of the explants.

Shoot proliferation

Well-established organogenic callus (7-week-old) obtained from callus induction treatments were used for further shoot regeneration. The *in vitro* induced and matured callus maintained in combination of BAP and NAA ((0.5,1.5), (0.5,2), (1,1), (1,1.5), (1,2), (1.5,1.5mg/l)) and ((0.5,2), (1,1), (1,1.5), (1,2), (1.5,1.5), (1.5,2mg/l)) for shoot tip and internode explants, respectively were excised and cultured on MS medium supplemented with various concentrations (0, 0.1, 0.5, 0.8, 1, 1.5mg/l) of BAP alone and (0, 1, 1.5, 2, 3mg/l) of BAP in combinations with (0.5mg/l) of NAA. Each jar contained three callus clumps, with three replicates for each treatment. After three weeks, the cultures were subcultured into fresh media to promote the further initiation of adventitious shoots. During this sub culturing process, any dead, dark brown cells were removed. All the cultures were kept at 25±2°C under dark conditions until the shoots arose from the calli. After six weeks, when shoots were regenerated well from calli, the cultures were kept in a growth room without direct contact with florescent light for one week and then transferred to a full photoperiod of 16/8 hours light/dark cycle at 2000-3000 lux light intensity with white fluorescent lights and relative humidity of 63%. After eight weeks, number of shoots per callus, length of shoots per callus and shoot regeneration percentage were recorded.

Shoot multiplication

Well-regenerated shoots were selected from the optimal regeneration medium to minimize the effects of varying media origins. The highly aseptic shoots, which exhibited the most prolific multiple shoot clusters, were carefully dissected into smaller segments at an angle. These segments were then transferred to a shoot multiplication MS medium, supplemented with different concentrations of BAP (0, 0.5, 1, 1.5, and 2 mg/l). Three shoots per culture jar and three replications for each

treatment were used. After four weeks multiplication rate of shoots was recorded.

Root induction

The elongated shoots, measuring 3-4 cm in length, were excised and cultured on half-strength MS medium supplemented with varying concentrations of IBA (0, 0.5, 1, 1.5, 2, and 3 mg/l) to induce root formation. Each treatment included three replicates, with three shoots cultured per jar. Root growth parameters were recorded after four weeks of culture.

Acclimatization

After successfully obtaining well-rooted in vitro propagated *P. edulis* plantlets measuring 3-5 cm in length, they were placed in culture vessels and allowed to acclimatize in a shaded area at room temperature for 5 days. On the evening of the fifth day, the plantlets were carefully removed from the culture vessels, and the root systems were rinsed under running tap water to eliminate any residual agar that could hinder nutrient absorption from the acclimatization substrates. The plantlets were kept in conditions of 80% relative humidity. Fourteen well-rooted shootlets (derived from shoot tip and internodal explants) were then transferred into plastic pots filled with a mixture of sterile river sand and forest soil in a 1:2 ratio. The pots were covered with transparent plastic bags that had random holes for air circulation, and drainage holes were drilled in the bottom of the pots. They were watered daily using a sprayer. After one week, the plastic covers were partially removed, and after two weeks, they were completely taken off.

Experimental design

The experiment was designed using a Completely Randomized Design (CRD) for all treatments. It involved various combinations and concentrations of plant growth regulators along with shoot tip and internode explants from the plant. The growth regulators constituted one factor, while the explants represented another factor. Each treatment included three replicates of culture jars, which served as the experimental units. Data collected from each trial were analyzed statistically using SAS software version 9.0 (SAS Institute, 2002). Mean differences were assessed using the LSD test at a 5% significance level. Analysis of variance was conducted for different traits, and means were compared using the least significant difference (LSD) method.

Results and Discussion

Surface sterilization of shoot tip and internode

ANOVA indicated that the concentration of NaOCl, the duration of explant exposure to the sterilant, and the interaction between NaOCl concentration and exposure time had a highly significant impact ($P < 0.001$) on the survival rate of aseptic explants.

The shoot tip and internode explants exhibited varying responses regarding sterilization percentages. The highest survival rate of clean and healthy explants ($92 \pm 1.15\%$) was observed when shoot tip explants were treated with 0.5% NaOCl for 10 minutes.

In contrast, internode explants showed their highest clean explant percentage ($83.33 \pm 0.67\%$) when treated with 1% NaOCl for the same duration. The lowest survival rates were noted for internode explants ($55.33 \pm 0.67\%$) at a 5-minute exposure to 0.5% NaOCl, while shoot tip explants had a survival rate of ($79 \pm 0.58\%$) when sterilized with 1% NaOCl for 15 minutes (Table 1).

In this study, the highest concentration (1%) of NaOCl applied for 10 and 15 minutes resulted in lower survival rates for shoot tip explants, likely due to the toxic effects and strong bleaching action of chlorine, which may have damaged the cells. Conversely, the lower concentration of NaOCl (0.5%) at 5 and 10 minutes led to low survival rates of internode explants, which were likely still highly contaminated. This could be attributed to the inadequate concentration and exposure time to effectively eliminate contaminants, as internodes are more mature and hairy.

Additionally, the differing sensitivities to toxicity between internodes and shoot tips may also play a role, along with varying contamination levels, with shoot tips generally showing less contamination. These findings align with research by Fufa *et al.*, (2019), which noted that optimal concentration and exposure times vary among different plants and plant parts based on their morphological characteristics, such as tissue softness or hardness. Similarly, Fanta *et al.*, (2018) reported that a 1% NaOCl solution for 10 minutes resulted in the highest percentage of contamination-free and surviving explants (87.5%) for Chankua and Lofuwa, and (83.3%) for Unnuka genotypes of *P. edulis* leaves. Based on the data presented in Table 1, using 0.5% NaOCl for 10 minutes for shoot tips and 1% NaOCl for 10 minutes for internodes proved to be the most effective sterilization treatments for establishing sterile cultures of *P. edulis*.

Callus Induction

Effect of 2, 4-D on Callus induction percentage (%), callus diameter (cm) and callus fresh weight (g)

ANOVA indicated a highly significant difference ($P < 0.001$) attributed to the main and the interaction effects of 2, 4-D concentrations and explants on the percentage of callus induction, callus diameter and callus fresh weight.

Different ranges and degrees of callus induction were observed in both shoot tip and internode explants after 30 days of culture on MS medium fortified with various concentrations of 2, 4-D, and the results were presented in Table 2. Among those concentrations, the best callus formation (100%) and (93.75 ± 6.25) was observed when MS medium was enhanced with 1.5 mg/l and 2 mg/l of 2,4-D for shoot tip and internodal explants, respectively. In contrast, the least amount of callus formation ($43.75 \pm 6.25\%$) was observed in internode explants grown on MS media enriched with 1 mg/l of 2,4-D. Meanwhile, shoot tip explants cultured on MS media with 1 mg/l and 2 mg/l of 2,4-D resulted in a callus induction percentage of 75%. Shoot tip explant treated with 2mg/l 2, 4-D showed declining callus induction percentage. The result of this study was consistent with the findings of Elangomathavan *et al.*, (2017) at high concentration of 2, 4-D the callus induction declined and turned hard texture with a brown color appearance observed which leads to necrosis later. This result was also observed by Amiri *et al.*, (2013) where the percentage of callus formation for *S.tuberosum* decreased as concentrations of 2, 4-D increased above 3mg/l. The same results were observed in the Phua *et al.*, (2016) investigation, where *C.nutans* leaf explants displayed a decreasing percentage of callus formation as the concentration of 2, 4-D increased above the optimum level. Shoot tip explants of the studied *P.edulis* treated with 1.5mg/l 2, 4-D followed by internode explants with 2mg/l 2, 4-D were the most effective treatments for callus induction (100%) and ($93.75 \pm 6.25\%$), respectively. It has been revealed from the present study that explant nature and concentrations are a significant feature and are responsible for the rate of achievement in callus induction.

The highest callus diameter (0.61 ± 0.005 cm) and (0.59 ± 0.005 cm) were obtained from internode and shoot tip explants on MS media enriched with 1.5 mg/l and 1 mg/l of 2, 4-D, respectively. Whereas, the lowest callus diameters (0.41 ± 0.006 cm) and (0.42 ± 0.005 cm) were

recorded for shoot tip and internode explants cultured on MS media supplemented with 2mg/l 2, 4-D (Table 2). The result of this study revealed that callus diameter decreased with the increase of 2, 4-D concentrations. The reason may be the herbicidal activity of 2, 4-D leads to the ceasing of the plant cell and callus tissue.

The highest callus fresh weight (0.65 ± 0.009 g) and (0.61 ± 0.009 g) was recorded on MS media supplemented with 1.5mg/l and 1mg/l of 2, 4-D for internode and shoot tip explants, respectively. Whereas, the lowest callus fresh weight (0.32g) was observed for shoot tip explant, followed by (0.4 ± 0.006 g) for internode explants induced on MS media containing 2mg/l 2,4-D. (Table 3).

The analyzed data showed that an increase in the concentration of 2, 4-D is associated with a decrease in callus fresh weight. The reason may be, herbaceous plants susceptible to the auxin herbicides do not exhibit sufficient detoxification, resulting in the prevention of normal nucleic acid metabolism and protein synthesis, leading to plant death (Phua *et al.*, 2016). According to Fanta *et al.*, (2018), the highest callus fresh weight (0.52g) was recorded by supplementing MS media with 1mg/l 2, 4-D for the lofuwa genotype of *P.edulis* from leaf-derived callus. Also, the minimum callus fresh weight was (0.19g) when MS media was supplemented with 2mg/l 2, 4-D. Thaniarasu *et al.*, (2016) also found that the highest fresh weight of callus (504.7 ± 1.33) and (323.8 ± 0.55 mg) was obtained from leaf and internodal explant of *P. bourneae* on MS media supplemented with 1mg/l 2, 4-D with 0.5mg/l BAP combination.

Effect of NAA combination with BAP on callus induction percentage (%), callus diameter (cm) and callus fresh weight (g)

In case of BAP and NAA combination treatments, ANOVA indicated a highly significant difference ($p < 0.01$) regarding the main effect of hormone concentrations and the interaction effect on callus induction percentage, callus diameter, and fresh weight of the callus.

Shoot tip explants cultured on MS medium with 1mg/l BAP+1.5mg/l NAA, 1mg/l BAP+2mg/l NAA and 1.5mg/l BAP+2mg/l NAA showed ($93.75 \pm 6.25\%$) callus induction percentage. The best callus induction ($93.75 \pm 6.25\%$) was also noted when the MS medium was enriched with a combination of 1mg/l and 1.5mg/l BAP with 2mg/l NAA for internodal explants. Whereas, the lowest percentage (25%) and ($18.75 \pm 6.25\%$) of

callus formation was recorded for shoot tip and internodal explants cultured on MS media supplemented with 0.5mg/l BAP with 1mg/l of NAA and 1.5mg/l of BAP with 1mg/l of NAA combination, respectively (Table 3). At lower concentrations of BAP and NAA combination (0.5mg/l BAP+1mg/l NAA) both the shoot tip and internode explants showed the lowest percentage (25%) of callus induction.

This result was supported by Aschale and Feyissa (2019) the maximum calli (100%) was recorded on MS medium supplemented with 1.5mg/l and 2mg/l NAA with 1mg/l and 0.5mg/l BAP in combination from *in vitro* leaf of *P.edulis* respectively. The results indicated that suitable levels and mixtures of cytokinins and auxins are crucial for generating callus in *P. edulis* (Fanta *et al.*, 2018; Aschale and Feyissa, 2019). Callus production efficiency of NAA and BAP is also seen in *O.aristatus* (Reshi *et al.*, 2013) and *O.stamineu* (Elangomathavan *et al.*, 2017).

Conversely, the MS medium that included only 2, 4-D, even at a low concentration, led to the development of callus (Table 2). Meanwhile, no callus formation was observed on both shoot tip and internode explants on BAP and NAA alone containing MS medium. This result was supported by the findings of Buko and Hvoslef-Eide (2020), meristems cultured on a basic MS media supplemented with all concentrations of BAP but without NAA did not induce callus in any of the varieties of sweet potato. The authors' findings indicated that BAP alone stimulated the formation of shoots instead of callus. The auxins (NAA) in combination with cytokinins (BAP) were found to be best for callus induction and growth in both shoot tip and internode explants investigated in this study.

The highest callus diameter of internode and shoot tip explants were (0.79±0.013cm) and (0.74±0.006cm) in the MS medium augmented with 1.5mg/l BAP together with 2mg/l NAA, respectively. Whereas, the lowest callus diameter (0.44±0.009cm) and (0.40±0.0025cm) were recorded for internode and shoot tip explants cultured on MS medium supplemented 0.5mg/l BAP in combination with 1mg/l NAA (Table 3). Concentrations of the hormone are the same.

The callus diameter of 2, 4-D alone treatments were smaller than that of callus diameters measured from BAP with NAA fortified MS medium (Compare tables 2 and 3). This may be due to the efficiency of the hormone in developing morphogenic callus. The result of this study revealed that callus diameter is directly related to callus

fresh weight. Taylor and Secor (1992) reported the same average tissue diameter was highly correlated with fresh weight over the range of callus size of potato protoplast-derived callus growth.

The highest callus fresh weight was obtained from both shoot tip (0.80±0.005g) and internode (0.8±0.01g) explants on MS media supplemented with 1.5mg/l BAP and 2mg/l NAA in combination. Whereas, the lowest callus fresh weight (0.07±0.003g) and (0.11±0.006g) was observed for shoot tip and internode explants on MS media containing 0.5mg/l of BAP in combination with 1mg/l of NAA, respectively (Table 3).

The result of this study revealed that the combination of BAP with NAA in different concentrations was more efficient for obtaining the high fresh weight of callus than 2, 4-D alone-treated treatments. The results of this study were supported by the findings of Thaniarasu *et al.*, (2016) who reported that the highest fresh weight was achieved on 0.5mg/l BAP combination with 1mg/l NAA in both leaf and internode explant of *P.bourneae*. According to Islam *et al.*, (2018), plant cells have their optimum hormone concentration to grow and increase the fresh weight of callus.

Morphological features of callus

MS media supplemented with different PGRs at different concentrations and combinations were able to induce callus for both shoot tip and internode explants of *P.edulis*. Callus was started from the cut end of explants, and gradually it covers the whole explant following mitosis division and results due to wound reaction or effect of exogenous growth regulator in the medium (Martin, 2002). Various colors of callus were observed ranging from colorless to yellowish and then light green. Their textures were either compact or friable depending on the explant type and hormone concentration as illustrated in table 5 and figure 1.

The findings of this study demonstrated that the combination of NAA and BAP treatments was the most effective for achieving a high frequency of greenish compact callus induction (Table 4). Earlier reports by Thaniarasu *et al.*, (2016) confirmed the same for *P.bourneae*. Oily and dark brown calli were observed across the two explants in all media supplemented with different concentrations of 2, 4-D. The callus obtained from this medium was not organogenic. The finding of Fanta *et al.*, (2018) was in line with this investigation on somatic embryogenesis of *P.edulis* from leaf explant.

The differences in texture and color of calli obtained on different media supplementations and genotypes were reported in *C.forskohlii* plants (Gopi and Mary, 2014). In this study, callus induced by 2, 4-D was not suitable for regeneration of plantlets whereas NAA and BAP combinations-based callus induction was suitable for shoot regeneration by producing greenish compact callus.

Shoot Proliferation

Shoot regeneration percentage (%)

The analysis of variance (ANOVA) showed that the percentage of shoot regeneration was significantly affected by the main effect of BAP concentrations and interaction effects ($p < 0.05$) on BAP treatment concentrations. However, the main effect of explants was not significantly affected ($p > 0.05$). In the case of BAP combination with (0.5mg/l) of NAA treatments, the ANOVA showed percentage of shoot regeneration was very highly significantly ($p < 0.001$) affected by the main effects of explants and PGRs concentrations. However, the interaction effects were not significantly affected ($p > 0.05$).

The greatest rate of shoot regeneration from callus ($66.67 \pm 8.33\%$) was recorded from callus derived from both shoot tips and internodes when cultured in MS medium enriched with 0.8 mg/l and 1 mg/l BAP, respectively. Whereas, the lowest percentage ($16.67 \pm 8.33\%$) and (25%) of shoot regenerations were recorded for shoot tip and internode-derived callus when cultured on MS medium enriched with 0.1 mg/l BAP and 0.5 mg/l BAP, respectively (Table 5).

Additionally, no shoot regeneration was observed on the control medium. At 0.1mg/l of BAP, no shoot regeneration was observed for internode explants. Increasing the BAP concentration in the medium enhanced the callus's ability to form shoots, as numerous shoot primordia were noted. Elangomathavan *et al.*, (2017) reported similarly that optimized BAP concentration led to an increase in shoot regeneration percentage but more increasing concentration of BAP resulted in a substantial reduction in regeneration efficiency. However, the result of this finding contradicts the report of Fufa *et al.*, (2019), the use of MS media supplemented solely with BAP did not promote shoot regeneration from the callus culture of *Jatropha*. This marks the first report on the regeneration of *P. edulis* utilizing BAP.

Based on the treatments of BAP combination with 0.5mg/l NAA, the highest percentage of shoot regeneration was observed from internodal explants (48.33%) followed by (31.67%) from shoot tip explants (Table 6). The reason may be due to the combination effect of BAP and NAA with endogenous hormones that may have a great impact on the shoot regeneration percentage of callus. Alternatively, it could be attributed to the effects of the photo-environment, including factors like temperature and light. Because calli were induced and grew under dark conditions, heating occurs due to the light source. The result of this study revealed that different explants have different regeneration efficiencies. The finding of Elangomathavan *et al.*, (2017) supports this result, who reported that leaf explants' regeneration efficiency was less compared to petiole and internode explants of *O.stamineus*.

With regards to the concentrations of BAP combinations with 0.5mg/l NAA, the highest percentage of shoot regeneration was recorded (66.67%) when MS media was supplemented with 2mg/l BAP+0.5mg/l NAA. Nonetheless, the lowest rate of shoot regeneration (33.33%) was observed when MS media were enriched with 1 mg/l BAP and 0.5 mg/l NAA (Table 6). In contrast with the result of Aschale and Feyissa (2019), the present result was increased by 3.71% by internode explants. The greatest shoot regeneration frequency (46.6%) was obtained from leaf-derived callus of *P. edulis* on MS medium supplemented with 0.1 mg/l BAP in combination with 0.5 mg/l thidiazuron.

During the process of tissue culture regeneration, the impacts of numerous elements including light, elicitors and PGRs; not only have an influence on the process of shoot regeneration and the production of biomass but also have an impact on the synthesis of a variety of bioactive compounds (Khan *et al.*, 2020).

Shoot number and shoot length

Analysis of variance revealed that both the number of shoots per callus and the length of shoots were highly significantly influenced ($P < 0.001$) by the main effects and interactions of BAP alone, as well as in combination with 0.5 mg/L NAA.

Internode callus grown on MS medium enriched with 1 mg/l BAP produced the greatest number of adventitious shoots, averaging 2.67 ± 0.083 . And (2.17 ± 0.083) per callus for shoot tip callus cultured on 0.8mg/l BAP were recorded.

Table.1 Various concentrations of sodium hypochlorite as a surface sterilant and different exposure durations were tested for their effects on the survival of aseptic shoot tip and internode explants of *P. edulis*.

Explant	NaOCl (%)	Time of exposure (minutes)	Survived aseptic explants (%)	Dead and Contaminated explants (%)
Shoot tip	0.5	5	86.67±2.4 ^{bc}	13.33±2.4 ^{fg}
		10	92±1.15 ^a	8±1.15 ^h
		15	89.33±0.67 ^{ab}	10.67±0.67 ^{gh}
	1	5	84±1.15 ^{cd}	16±1.15 ^{ef}
		10	83.33±0.67 ^d	16.67±0.67 ^e
		15	79±0.58 ^e	21±0.58 ^d
Internode	0.5	5	55.33±0.67 ^h	44.67±0.67 ^a
		10	57.67±1.45 ^h	42.33±1.45 ^a
		15	66±0.00 ^g	34±0 ^b
	1	5	73.33±0.67 ^f	26.67±0.67 ^c
		10	83.33±0.67 ^d	16.67±0.67 ^e
		15	81.67±0.33 ^{de}	18.33±0.33 ^{de}
CV (%)			2.34	8.13
LSD (5%)			3.06	3.06

Values correspond to means±Se. Mean followed by the same letter (s) in the columns that share the same designation do not show significant differences at the 5% significance level. CV stands for Coefficient of Variation, while LSD refers to Least Significant Difference.

Table.2 Interaction effect of explants and 2, 4-D concentrations on callus fresh weight (CFW), callus diameter (CDT) and callus induction percentage (CIP) of *P.edulis* from shoot tip and internode explants.

Explant	2, 4-D (mg/l)	CFW (g)	CDT (cm)	CIP (%)
Shoot tip	0	0±0 ^g	0±0 ^f	0±0 ^e
	1	0.61±0.009 ^b	0.59±0.005 ^b	75±0 ^c
	1.5	0.56±0.003 ^d	0.51±0.005 ^d	100±0 ^a
	2	0.32±0.011 ^f	0.41±0.006 ^e	75±10.21 ^c
Internode	0	0±0 ^g	0±0 ^f	0±0 ^e
	1	0.58±0.007 ^c	0.56±0.013 ^c	43.75±6.25 ^d
	1.5	0.65±0.009 ^a	0.61±0.005 ^a	81.25±6.25 ^{bc}
	2	0.4±0.006 ^e	0.42±0.005 ^e	93.75±6.25 ^{ab}
CV (%)		3.53	3.17	17.95
LSD (5%)		0.02	0.018	15.35

Values correspond to means±Se (Standard error). Mean values followed by the same letter (s) in the columns that share the same designation do not show significant differences at the 5% significance level. CV stands for Coefficient of Variation, while LSD refers to Least Significant Difference.

Table.3 Interaction effect of different concentrations and combination of BAP and NAA on fresh weight of callus (FWC), callus diameter (CDT) and callus induction percentage (CIP) of *P.edulis*.

Explant	BAP (mg/l)	NAA (mg/l)	FWC(mg)	CDT(cm)	CIP (%)
Shoot tip	0	0	0±0 ^m	0±0 ^l	0±0 ^g
	0	1	0±0 ^m	0±0 ^l	0±0 ^g
	0	1.5	0±0 ^m	0±0 ^l	0±0 ^g
	0	2	0±0 ^m	0±0 ^l	0±0 ^g
	0.5	0	0±0 ^m	0±0 ^l	0±0 ^g
	0.5	1	0.07±0.003 ^l	0.40±0.0025 ^k	25±0 ^{ef}
	0.5	1.5	0.11±0.003 ^k	0.49±0.013 ^{hi}	50±0 ^d
	0.5	2	0.18±0.003 ^j	0.50±0.005 ^{hi}	87.5±7.22 ^{ab}
	1	0	0±0 ^m	0±0 ^l	0±0 ^g
	1	1	0.46±0.004 ^g	0.54±0.013 ^g	81.25±6.25 ^b
	1	1.5	0.6±0.006 ^c	0.6±0.029 ^e	93.75±6.25 ^a
	1	2	0.51±0.009 ^e	0.57±0.011 ^f	93.75±6.25 ^a
	1.5	0	0±0 ^m	0±0 ^l	0±0 ^g
	1.5	1	0±0 ^m	0±0 ^l	0±0 ^g
	1.5	1.5	0.5±0.009 ^d	0.72±0.011 ^c	87.5±7.22 ^{ab}
	1.5	2	0.80±0.005 ^a	0.74±0.006 ^{bc}	93.75±6.25 ^a
Internode	0	0	0±0 ^m	0±0 ^l	0±0 ^g
	0	1	0±0 ^m	0±0 ^l	0±0 ^g
	0	1.5	0±0 ^m	0±0 ^l	0±0 ^g
	0	2	0±0 ^m	0±0 ^l	0±0 ^g
	0.5	0	0±0 ^m	0±0 ^l	0±0 ^g
	0.5	1	0.11±0.006 ^k	0.44±0.009 ^j	25±0 ^{ef}
	0.5	1.5	0.26±0.005 ⁱ	0.48±0.010 ⁱ	31.25±6.25 ^c
	0.5	2	0.30±0.006 ^h	0.50±0.006 ^h	62.5±7.22 ^c
	1	0	0±0 ^m	0±0 ^l	0±0 ^g
	1	1	0.49±0.011 ^f	0.54±0.013 ^g	68.75±6.25 ^c
	1	1.5	0.53±0.010 ^d	0.62±0.012 ^e	87.5±7.22 ^{ab}
	1	2	0.64±0.006 ^b	0.65±0.02 ^d	93.75±6.25 ^a
	1.5	0	0±0 ^m	0±0 ^l	0±0 ^g
	1.5	1	0.54±0.013 ^d	0.74±0.016 ^{bc}	18.75±6.25 ^f
	1.5	1.5	0.61±0.011 ^c	0.76±0.014 ^b	87.5±7.22 ^{ab}
	1.5	2	0.8±0.01 ^a	0.79±0.013 ^a	93.75±6.25 ^a
CV (%)			4.84	6.2	23.69
LSD (5%)			0.016	0.03	12.28

Values correspond to means±SE (Standard error). Mean values followed by the same letter (s) in the columns that share the same designation do not show significant differences at the 5% significance level. CV stands for Coefficient of Variation, while LSD refers to Least Significant Difference.

Table.4 Effect of different concentrations of auxins and cytokinins ratio on morphological features of callus induced from shoot tip and internode explants of *P.edulis*

Plant growth regulators (mg/l)			Type of explant	
			Shoot tip	Internode
2, 4-D	BAP	NAA	Texture and nature of callus	Texture and nature of callus
1	-	-	whitish, fragile	brownish, fragile
1.5	-	-	whitish, fragile	blue, less compact
2	-	-	white, slimy	white, slimy
0.5	1		brownish, fragile	brownish, fragile
0.5	1.5		whitish, fragile	brownish, fragile
0.5	2		whitish, less compact	light green, less compact
1	1		light green, compact	light green, compact
1	1.5		greenish, compact	greenish, compact
1	2		dark green, compact	dark green, compact
1.5	1		no callus formation	whitish, fragile
1.5	1.5		greenish, compact	greenish, compact
1.5	2		whitish, nodular	light green, compact

Table.5 Interaction effects of explants and BAP concentrations on the percentage of shoot regeneration (SRP).

Explant	BAP (mg/l)	SRP (%)
Shoot tip	0	0±0 ^e
	0.1	16.67±8.33 ^{de}
	0.5	25±0 ^{cd}
	0.8	66.67±8.33 ^a
	1	50±0 ^{ab}
	1.5	41.67±8.33 ^{bc}
Internode	0	0±0 ^e
	0.1	0±0 ^e
	0.5	25±0 ^{cd}
	0.8	41.67±8.33 ^{bc}
	1	66.67±8.33 ^a
	1.5	41.67±8.33 ^{bc}
CV (%)		32.66
LSD (5%)		17.12

Values correspond to means±SE (Standard error). Mean values followed by the same letter (s) in the columns that share the same designation do not show significant differences at the 5% significance level. CV stands for Coefficient of Variation, while LSD refers to Least Significant Difference.

Table.6 Main effects of explant and BAP with 0.5mg/l NAA combination on percentage of shoot regeneration (SRP).

Explant	SRP (%)
Shoot tip	31.67 ^b
Internode	48.33 ^a
LSD (5%)	7.77
BAP (mg/l)	SRP
0	0 ^d
1	33.33 ^c
1.5	54.17 ^b
2	66.67 ^a
3	45.83 ^b
LSD (5%)	12.29
CV (%)	25.52

Mean values followed by the columns that share the same designation do not show significant differences at the 5% significance level. CV stands for Coefficient of Variation, while LSD refers to Least Significant Difference.

Table.7 Interaction effect of explants and BAP concentrations on number of shoots (NST) and length of shoot (STL) of *P.edulis*.

Explant	BAP(mg/l)	NST	STL (cm)
Shoot tip	0	0±0 ^f	0±0 ^h
	0.1	0.25±0.00 ^f	0.97±0.033 ^g
	0.5	0.94±0.241 ^e	1.67±0.00 ^f
	0.8	2.17±0.083 ^b	1.71±0.007 ^e
	1	2.08±0.083 ^b	1.92±0.003 ^c
	1.5	1.75±0 ^c	1.61±0.007 ^f
Internode	0	0±0 ^f	0±0 ^h
	0.1	0±0 ^f	0±0 ^h
	0.5	1.25±0 ^d	1.82±0 ^d
	0.8	2.08±0.083 ^b	2.13±0.003 ^b
	1	2.67±0.083 ^a	2.5±0 ^a
	1.5	2.08±0.083 ^b	2.13±0.003 ^b
CV (%)		11.97	1.77
LSD (5%)		0.26	0.04

Values correspond to means±SE (Standard error). Mean values followed by the columns that share the same designation do not show significant differences at the 5% significance level. CV stands for Coefficient of Variation, while LSD refers to Least Significant Difference.

Table.8 Interaction effect of explants and BAP with 0.5mg/l NAA treatments on number of shoots (NST) and length of the shoot (STL) of *P.edulis*.

Explants	BAP(mg/l)	NST	STL(cm)
Shoot tip	0	0±0 ^f	0±0 ^g
	1	2.44±0.113 ^c	1.5±0.06 ^f
	1.5	3±0 ^b	1.87±0.07 ^e
	2	2±0.19 ^{cd}	2.47±0.07 ^b
	3	1.33±0 ^e	2.2±0 ^d
Internode	0	0±0 ^f	0±0 ^g
	1	1.78±0.11 ^{de}	1.93±0.03 ^e
	1.5	3.11±0.44 ^{ab}	2.33±0.03 ^c
	2	3.56±0.113 ^a	2.87±0.07 ^a
	3	2.11±0.11 ^{cd}	2.5±0 ^b
CV (%)		15.06	4.26
LSD (5%)		0.49	0.13

Values correspond to means ± SE (Standard error). Mean values followed by the columns that share the same designation do not show significant differences at the 5% significance level. CV stands for Coefficient of Variation, while LSD refers to Least Significant Difference.

Table.9 Shoot multiplication rate over 4 weeks of cultures of *P.edulis* from shoot tip and internode-derived callus shoots.

Explant	BAP (mg/l)	Number of shoot buds		Multiplication rate (%)
		At beginning	After 4 weeks	
Shoot tip	0	9	23	2.56
	0.5	9	48	5.33
	1	9	73	8.11
	1.5	9	85	9.44
	2	9	78	8.67
Internode	0	9	25	2.78
	0.5	9	49	5.44
	1	9	75	8.33
	1.5	9	89	9.89
	2	9	77	8.56

Table.10 The main effect of explants and IBA concentrations on root number and root induction percentage of *P.edulis* shoots.

Explant Internode Shoot tip LSD (5%)	Root number 7.34 ^a 7.02 ^b 0.3	
IBA (mg/l)	Root number	Root induction percentage (%)
0	3.5 ^c	29.17 ^d
0.5	5.22 ^d	58.33 ^c
1	7.39 ^c	83.33 ^b
1.5	9.06 ^{ab}	100 ^a
2	9.34 ^a	66.67 ^c
3	8.56 ^b	37.5 ^d
CV (%)	6.1	20
LSD (5%)	0.52	14.89

Mean values followed by the same letter(s) in the columns that share the same designation do not show significant differences at the 5% significance level. CV stands for Coefficient of Variation, while LSD refers to Least Significant Difference.

Figure.1 Callus morphology: A) compact callus, B) friable callus

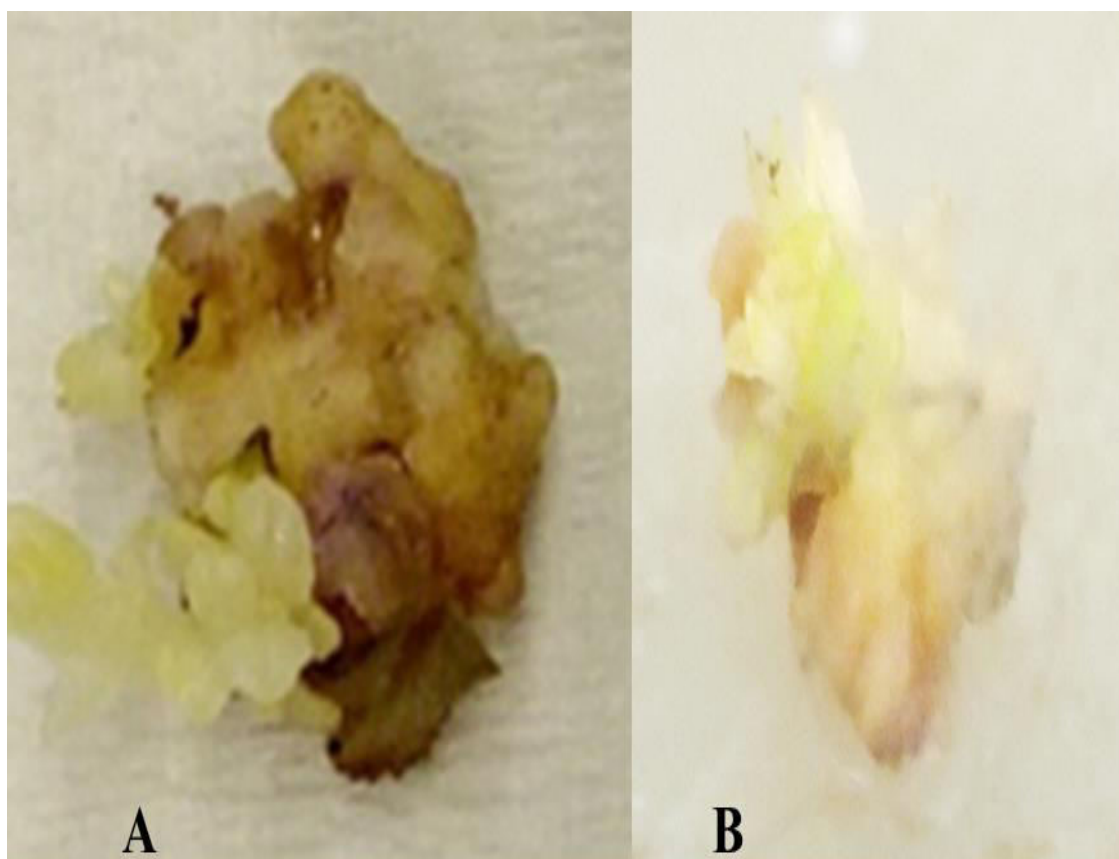


Figure.2 Root length variation across IBA concentrations. Bars with different letters differ significantly ($p < 0.05$, LSD test).

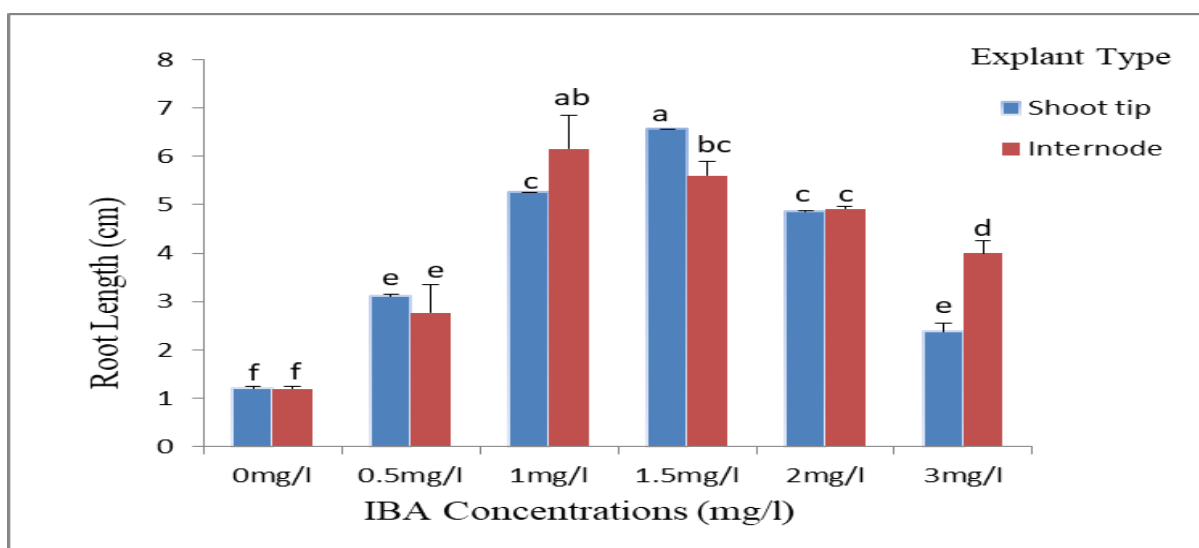


Figure.3 Wild-type controls (A) Vs. Acclimatized plantlets (B)



The lowest numbers of shoots 0.25 for shoot tip and 1.25 for internode explants were observed when callus was cultured on MS medium containing 0.1 mg/L and 0.5 mg/L BAP, respectively (Table 7). Notably, shoot production per callus increased with higher concentrations of BAP, ranging from 0.1 to 0.8 mg/l for shoot tip explants and from 0.1 to 1 mg/L for internode explants. Internode explants at the concentration of 0.1mg/l BAP cannot regenerate any shoot. This may be due to the insufficient amount of BAP concentration to induce shoots.

With regards to BAP combination with 0.5 mg/l NAA treatments, the highest number of regenerated shoots (3.56 ± 0.113) was recorded from internode-derived callus

on MS media supplemented with 2mg/l BAP with 0.5mg/l NAA combination. In the case of shoot tip explant the highest number of shoots regenerated (3 ± 0.0) were recorded when MS media was supplemented with 1.5mg/l BAP with 0.5mg/l NAA. The minimum shoot regeneration 1.33 ± 0.00 from shoot tip and 1.78 ± 0.11 from internode callus was observed when cultured on MS medium supplemented with 3 mg/L and 1 mg/L BAP in combination with 0.5 mg/l NAA, respectively (Table 8).

In this experiment, the maximum shoot number for both shoot tip and internode callus was recorded on the media containing BAP with NAA with combination rather than BAP alone. The reason may be the synergistic effect of

cytokinins and auxin in the medium could enhance the rate of shoot multiplication and it has been reported by (Shekhawat *et al.*, 2015). Shoots regenerated on BAP alone MS media form necrosis at the base and it causes death.

This may be due to the effects of hormone concentrations from callus media. This means there is a transfer of hormones from callus formation to callus regeneration media via plant tissue. Aschale and Feyissa (2019) reported that the highest mean shoot number per callus (1.66 ± 1.04) was obtained on MS medium supplemented with 1.5mg/l BAP+1.0mg/l thidiazuron.

The highest shoot length from regenerated shoots (2.5 ± 0.00 cm) and (1.92 ± 0.003 cm) was observed from internode and shoot tip explants respectively on MS medium supplemented with 1mg/l BAP. The same hormone concentrations were found for both shoot tip and internode explants.

Whereas, the lowest shoot length (0.97 ± 0.003) and (1.82 ± 0.00 cm) of regenerated shoots were recorded for shoot tip and internode explants when the callus was grown on MS medium enriched with 0.1 mg/L and 0.5 mg/L BAP, respectively (Table 7). The result revealed that BAP at the lowest concentration shoot length was smaller. The reason may be the insufficiency of BAP to develop and elongate shoots.

Concerning BAP with NAA combination, the highest shoot lengths (2.47 ± 0.07 cm) and (2.87 ± 0.07 cm) from regenerated shoots were observed from shoot tip and internode explants on MS media supplemented with 2mg/l BAP+0.5mg/l NAA. Conversely, the shortest shoot length for both explants was recorded on MS medium supplemented with 1 mg/l BAP and 0.5 mg/L NAA. The minimum shoot length of regenerated shoot from internode explants was (1.93 ± 0.03 cm) and (1.5 ± 0.06 cm) for shoot tip explants (Table 8). The result of this study revealed that the concentration of BAP increases (0.1-1mg/l) shoot length also increases. Aschale and Feyissa (2019) pointed out that the maximum mean (0.63 ± 0.19 cm) shoot length was obtained on MS medium supplemented with 0.5mg/l BAP+0.1mg/l thidiazuron from leaf-derived callus of *P.edulis*. The variation may be the effect of thidiazuron. The biological effect of thidiazuron is very variable and is determined by factors such as the concentration of the compound, the amount of time it is exposed, the kind of explant that is utilized and the genotype of the plant (Cosic *et al.*, 2015).

Shoot Multiplication

Multiplication rate of shootlet per explant

A vital component affecting the efficiency of the micropropagation system is the rate of multiplication. This can be calculated as the ratio of shoot number at the end of the subculture to the initial number of shoots (Mendes *et al.*, 1999). The formations of adventitious buds of *P.edulis* on all explants at both the callus induction, shoot regeneration and the multiplication stages were observed. The multiplication rate was calculated for the multiplication stage of *P.edulis*. The multiplication rate of *P.edulis* in this research was occasionally very high (up to 89 shoots) depending on the BAP concentration in the medium. The highest rate of multiplication (9.89) was obtained from internode explants and (9.44) for shoot tip explants treated with 1.5mg/l of BAP. Whereas, the rate of shoot multiplication was minimal at hormone-free MS medium for both shoot tip and internode explants (Table 9). An increase in the concentration of BAP (0-1.5mg/l) led to an increase in the multiplication rate. The result of this study was consistent with the findings of Khalafalla *et al.*, (2011) who reported that the multiplication rate of *B.senegalensis* increase with the concentration of cytokinin also increases. SK (2000) also obtained the highest rate of multiplication (6.31) on 4-year-old elite *E.tereticornis* trees.

Rooting

Root induction percentage (%)

ANOVA results indicated a highly significant effect ($p < 0.001$) of IBA concentration on the percentage of root formation. In contrast, neither the main effect of the explant type nor the interaction between explant and IBA concentration showed statistical significance ($p > 0.05$).

The maximum rooting rate of 100% was achieved on MS medium containing 1.5 mg/L IBA, with the second-highest rate of 83.33% observed at a concentration of 1 mg/L IBA. The lowest rooting percentage (29.17%) was obtained on MS media without IBA (Table 10). According to Tsegaw and Feyissa (2014) and Aschale and Feyissa (2019), complete (100%) rooting of *P. edulis* was achieved on MS medium supplemented with 1.0 mg/L IBA from meristem shoots and leaf-derived callus shoots, respectively on half-strength MS medium. Kebede and Abera (2014) also reported that (97.00 ± 0.28) was obtained on a half-strength MS medium at 2mg/l of

IBA. These observations in our study were also similar to the findings on *P. ambonicus* by Ab Rahman *et al.*, (2015) who reported high root proliferation per explant obtained on MS medium containing 1mg/l IBA. Among the given concentrations IBA with the highest concentrations resulted in a reduction in rooting percentages.

Root number per explant

The ANOVA results revealed a significant influence of the main factors ($P < 0.05$) on the number of roots per shoot, while the interaction between these factors was not statistically significant ($P > 0.05$).

The maximum root number (7.34) was recorded for the internode followed by (7.02) for shoot tip explants (Table 10). The difference may come from the endogenous hormone levels of explants. The highest number of roots (9.34) was observed in shoots cultured on MS medium enriched with 2 mg/L of IBA. The lowest number (3.5) of roots was recorded on IBA-free MS media (Table 10). Rooting of treatments treated without IBA may be due to the existence of endogenous hormones inside the shoots. Shoot organogenesis is promoted by the interaction between naturally occurring endogenous hormones within plant tissues and externally applied exogenous hormones. Consequently, the concentration of endogenous hormones in cultured explants may play a pivotal role in initiating shoot formation (Lee and Huang, 2013; Elangomathavan *et al.*, 2017). Kebede and Abera (2014) reported that the highest mean numbers (32.73 ± 0.14) of roots were obtained from 2mg/l IBA-supplemented MS media of *P. edulis*. All treatment concentrations of IBA enhance the root initiation including hormone free. Similar *in vitro* root initiation was observed in *M. piperita* (Islam *et al.*, 2018), and *P. edulis* (Aschale and Feyissa, 2019).

Root length per explant

The length of roots generated in half-strength MS medium was highly significantly influenced ($p < 0.01$) by the main effects of explant and IBA concentration, as well as their interaction.

The longest roots (6.56 ± 0.00 cm) were observed in shoot tip explants cultured on half-strength MS medium containing 1.5 mg/l of IBA, followed by internode explants (6.16 ± 0.68 cm) grown on medium supplemented with 1 mg/L of IBA. Meanwhile, the minimum root length (1.12 ± 0.04 cm) was obtained for

both shoot tip and internode explants on IBA-free half MS media (Figure 2). Most of the time the number of root and root lengths can't be obtained at the same concentration. The highest root length (3.9 ± 0.25) was achieved on MS-supplemented 0.5mg/l IBA but the highest root number (16.3 ± 0.23) was obtained from 1mg/l IBA-supplemented MS media (Islam *et al.*, 2018). This might be due to the activity of roots to absorb water and minerals at lower IBA concentrations. Aschale and Feyissa, (2019) finding also in agreement with this justification, who states that the highest mean number of roots per explant (10.15 ± 0.95) and mean length (5.06 ± 0.35 cm), was obtained on MS medium supplemented with 2.0 and 0.6mg/l IBA, respectively from *in vitro* leaf derived callus shoots of *P. edulis*.

Acclimatization of Plantlets

Shoots generated through *in vitro* culture are highly sensitive and vulnerable to abrupt changes in their surroundings, which can lead to damage if they are not gradually conditioned to external environments. Therefore, the process of acclimatization is crucial for ensuring successful adaptation of rooted plantlets to natural *ex vitro* conditions. Therefore, plantlets from indirect *in vitro* regeneration of *P. edulis* were transferred to a shed house at Hawassa University College of Agriculture for acclimatization (Figure 3). A total of 28 plantlets were subjected to acclimatization (14 for shoot tip and 14 for internode-derived plantlets). Among these 8 plantlets survived for each shoot tip and internode explant-derived plantlets on forest topsoil and river sand at a ratio of 2:1. This means 57.14 percent of survival was obtained from each explant. 42.86 percent of plantlets were lost. The loss of plantlets could be attributed to stress induced by exposure to a new environment. Among those stresses the high temperature inside the shed house in the daytime leads to excessive water loss and death. As reported by Aschale and Feyissa (2019), leaf-derived callus plantlets of *P. edulis* exhibited a survival rate of 78% under both glasshouse and outdoor conditions on red soil, sand and compost in a 2:1:1 ratio respectively. Fanta *et al.*, (2018) also obtained 92%, 88% and 80% survived frequency for Chankua, Unnuka and Lofuwa respectively from leaf-derived embryogenic callus plantlets on sterilized river sand and soil at 2:1 ratios at Holeta Agricultural Research Center.

In conclusion, *P. edulis* is the indigenous and underutilized tuber crop species in Ethiopia. Due to its enormous potential for household consumption and marketing, the cultivation and propagation of this crop

are of significant importance; however, traditional methods fall short in achieving optimal productivity. Advancements such as plant tissue culture and genetic transformation offer accelerated approaches, contingent upon the development of robust regeneration protocols. This study successfully established an efficient indirect organogenesis protocol aimed at facilitating large-scale propagation, supporting breeding initiatives, and conserving the germplasm of *P. edulis*. This protocol enables mass propagation and paves the way for synthetic seed production, *cryopreservation*, and metabolite studies. Future work should test its efficacy across diverse *P. edulis* accessions. In addition to that molecular markers should utilize to detect somaclonal variation and verify the genetic precision of the *in vitro* regenerated plants.

Author Contributions

Temare Mossie Amanie: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. Bizuayehu Tesfaye: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Conflicts of Interest

The authors declare no conflict of interest.

Consent for publication: Not applicable.

Data availability statement

The data presented in this study are available on request from the corresponding authors.

Additional information

No additional information is available for this paper.

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