Indirect somatic embryogenesis and plant regeneration through leaf and nodal cultures of *Piper longum* L.

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This research aims to develop a protocol for inducing somatic embryogenesis and plant regeneration through callus in *Piper longum*. Leaf and nodal explants were cultured in the Murashige and Skoog (MS) medium added with 2, 4-dichlorophenoxyacetic acid (2, 4-D) or α-naphthaleneacetic acid (NAA), Kinetin (KN), and 10% coconut water (CW). The maximum frequency of embryogenic/nodular callus development (66.66%) and the number of embryos (28.33±3.511) per 0.2-0.3 g embryogenic callus developed from the leaf explant was obtained in the MS medium fortified with 1.5 mg/L 2, 4-D, 1.0 mg/L KN, & 10% CW. Similarly, the maximum frequency of embryogenic/nodular callus production (50%) and the number of embryos (12.66±2.51) per 0.2-0.3 g embryogenic callus developed from the stem explant were obtained in the MS + 1.0 mg/L NAA + 10% CW. Somatic embryo differentiation, maturation, and conversion were obtained when the nodular calli with various stages of embryos were transferred to the MS + 0.25 -1.5 mg/L thidiazuron + 10% CW. Somatic embryos were also transformed into seedlings after being transplanted to the MS media with no growth regulators. This study developed a technique for micropropagation of *P. longum* using somatic embryos derived from leaf and nodal explants, which could serve as the foundation for an alternate method of micropropagation and *ex-situ* germplasm conservation.

Keywords: Coconut water, micropropagation, Murashige and Skoog medium, nodular callus, somatic embryos

An alternate method for micropropagation in plant species is the asexual embryogenesis of somatic cells due to the totipotent nature of plant cells through the tissue culture technique. Bipolar somatic embryos, which resemble zygotic embryos morphologically, are produced through direct or indirect somatic embryogenesis.

Induction of somatic embryos for micropropagation through callus culture and other explants has been investigated in some medicinal plants including *Piper nigrum* (Joseph *et al*., 1996; Nair & Gupta, 2003, 2006; Sasi & Bhat, 2016), *P. colubrinum* (Yusuf *et al*., 2001), and *P. aduncum* (De Sousa *et al*., 2020).

The callus is a homogeneous mass of undifferentiated cells that has the biological capacity to re-differentiate into somatic embryos, shoots, or roots under suitable conditions in a culture medium (Adhikari & Pant, 2013; Pant, 2014). In culture media, plant tissue induces several forms of callus, including compact, friable, embryogenic, and non-embryogenic callus. Among these, embryogenic callus is important for the development of somatic embryos. Several factors may affect the form of callus generated in tissue culture, such as the kind and amount of plant growth regulators (auxins and cytokinins) employed in the culture media, the genetic makeup of the plant, and the culture environment such as light intensity, temperature,

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etc. (Bhatia, 2015). According to Santos *et al.* (2002), somatic embryogenesis is one of the most crucial ways to propagate superior or genetically modified plants in large quantities and also serves as a helpful experimental model to study the processes of plant embryogenesis. It has the potential for automating the large-scale generation of embryos in bioreactors, and the ability to sow artificial seeds in the wild (Giri *et al.*, 2004). Similarly, tissue culture techniques have the potential to boost vegetative propagation rates and maintain pathogen-free plants (Saito & Nakano, 2002). Moreover, tissue culture is an excellent approach for the ex-situ conservation of plant biodiversity because it permits the rapid vegetative reproduction and safeguarding of several uncommon, susceptible, vulnerable, and fragile plant species from a relatively small tissue or part of plants (Fay, 1992, 1994; Rao, 2004; Joshi *et al.*, 2022; Pandey *et al.*, 2023).

Since ancient times, *P. longum*, a tropical and subtropical medicinal plant, has been used to treat cough, bronchitis, stomachaches, sleeplessness, and diabetes (IUCN, 2004; Thapa, 2020; Thapa *et al*., 2023). It contains the alkaloid piperin, which has digestion-improving properties, anti-mutagenic and anti-cancer properties, and anti-diarrheal and anti-dysenteric properties (Choudhary & Singh, 2018). It is distributed throughout the world, including India, Bhutan, Sri Lanka, Malaysia, Indonesia, Nepal, Singapore, and Burma. The Nepalese government has prioritized *P. longum* for scientific and technological advancement, in addition to agricultural technology development (DPR, 2012, 2017). It requires *ex-situ* conservation since people heavily utilize its fruits and roots for a variety of diseases, reducing its population in its native habitats. However, micropropagation through nodal explant is difficult in *P. longum* because of the frequent contamination of culture by endophytic bacteria (Bhat *et al*., 1995; Parida & Dhal, 2011; Sathelly *et al*., 2016). Somatic embryogenesis through callus culture of the leaf may therefore be useful for this purpose. This study attempts to develop an effective protocol for the production of somatic embryos from leaf and nodal explants for micropropagation. This is the first study that has developed a protocol for indirect somatic embryogenesis using leaf and nodal segments in *P. longum*.

Materials and methods

Study area

The study was carried out in the Rupandehi district situated in the southern part of western Nepal (see Figure 1). The district is located between 27° 20' 00" N–28° 47' 25" N latitudes and between 83° 12' 16" E–83° 38' 16" E longitudes. The elevation of the terrain ranges from 100 m to 1229 m above the mean sea level (msl). It is bounded by Nawalparasi, Kapilvastu, and Palpa districts on the east, west, and north, respectively, and India on the south. The district, moreover, experiences a sub-tropical type of climate, and so the plants found there are of tropical to sub-tropical in nature. The study was conducted from 2022 to 2023.

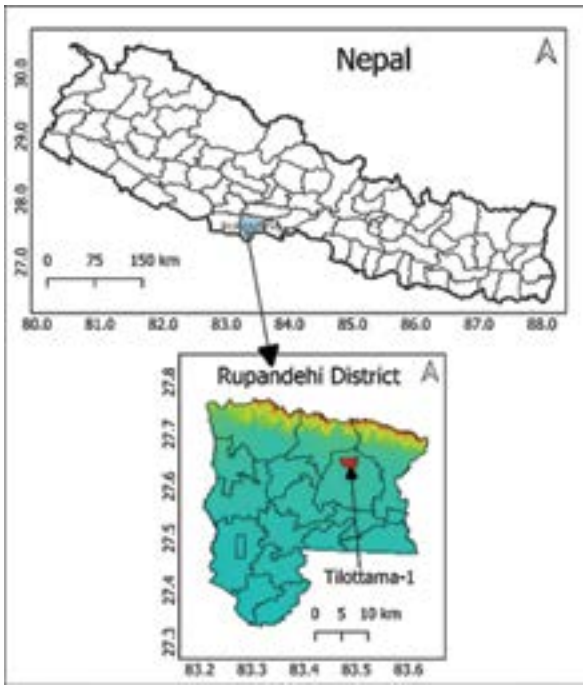

Figure 1: Location of the study area in the map of Nepal and the site of collection of plant material.

Collection of plant materials

P. longum was collected from the Rupandehi district of western Nepal in October-November 2022 at the elevation of 160 m above the mean

sea level. The plant specimens were identified by comparing with the herbarium specimens of *P. longum* deposited at the National Herbarium and Plant Laboratories (KATH) and its voucher specimen (Voucher No. 135, 136) were deposited at the Tribhuvan University Central Herbarium (TUCH), Kirtipur. A few plants were cultivated in the Botanical Garden of the Central Department of Botany, Tribhuvan University.

Surface sterilization of plant materials

The leaves and nodal segments (stems) were taken from the garden-grown *P. longum* plants from the Botanical Garden, which were immersed in Tween-20 (2% v/v) for 1/2 an hour before being cleaned with the running tap water. The nodal segment was immersed in 0.2% Bavistin (w/v) for one hour, 70% alcohol (v/v) for one minute, and 0.1% $HgCl_2$ (w/v) for seven minutes while the leaves were dipped in 0.1% $HgCl_2$ for three minutes and 70% alcohol for thirty seconds. Following sterilization, the leaves and nodal segments were washed three times with sterilized pure water to remove any mercuric chloride residues..

Inoculation of explants and embryogenic callus induction

The nodal (0.5 cm) and leaf (0.5 cm^2) explants were cut off and cultured on the MS medium (Murashige and Skoog, 1962) lacking plant growth regulators (PGRs) and coconut water (CW), and the MS medium added with PGRs, such as 0.25–4.5 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) alone, 0.25–4.5 mg/L α-naphthalene acetic acid (NAA) alone, and in combination with 0.25–3.5 mg/L 2, 4-D+0.25mg/L to 1.0 mg/L kinetin (KN), 10% CW (v/v), 3% sucrose (w/v), and 0.8% agar (w/v). Before adding agar, the pH of the media was maintained at 5.8, sterilized in autoclave for 15 minutes at 121°C temperature and 15-pound pressure, and then poured into the sterile 78 mm × 122 mm Jars (30 mL each) covered with aluminum foil. Cultures were maintained at 25± 2ºC in a culture room with a 16/8 hours light-dark cycle utilizing cool-white fluorescent lamps (28-42 μmol/m^2/s).

Somatic embryo differentiation, development, and maturation

For somatic embryo (SE) differentiation, growth, and maturation, the calli developed from both the leaves and nodal explants were transferred to the MS media without PGRs and CW and added with 0.25 to 3.5 mg/L thidiazuron (TDZ) and 10% CW. The cultures were maintained at 25±2°C with cool-white fluorescent light (3000 lux) for 16 hours. At the intervals of 30 days, subcultures were performed into new media. The SEs that had formed on the callus surfaces of each subculture were separated and transferred to full-strength, 1/2-strength, and 1/4-strength MS media. The MS media (without PGRs) were solidified with 0.8% agar to facilitate the germination or embryo conversion into seedlings.

Histological studies

The embryogenic calli and somatic embryos were dipped in 1% acetocarmine for 12 hours, and then heated in test tubes until boiling, squashed in glass slides, and observed under a compound microscope. Similarly, the serial thin sections of the embryogenic calli and morphogenic calli were cut with a fine blade, stained with safranin for two minutes, mounted with 10% glycerin water, and examined under a compound microscope. Photographs were taken using the ScopeImage 9.0 image-processing software professionally designed for digital microscope (LABOMED-121, INC., USA).

Statistical analysis

The frequency of the embryogenic/nodular callus produced per 0.2 g to 0.3 g callus and the number of somatic embryos produced was calculated in terms of the percentage of six replicates, and the standard deviation (SD) was calculated. Moreover, the frequency of embryogenic/nodular calli that developed into shoots, roots, calli, and whole plantlets was expressed in terms of percentage (%).

Results

Embryogenic and nodular callus induction

The formation of embryogenic callus from explants is the first stage in the growth and development of somatic embryos. In our study, the embryogenic and nodular calli were not induced in MS media without the addition of PGRs and coconut water. However, the MS media supplemented with 2, 4-D alone, NAA alone, or in combination with 2, 4-D and KN, and 10% CW, leaf, and nodal segments developed embryogenic mass of callus initially in *P. longum* (Figures 2A, 2B). In a subsequent subculture using the same media, the embryogenic callus changed into a nodular aggregate callus with various stages of somatic embryos (Figure 2D); however, in the case of nodal segments, the nodular callus was produced by subculturing the embryogenic callus at dark (Figure 2C).

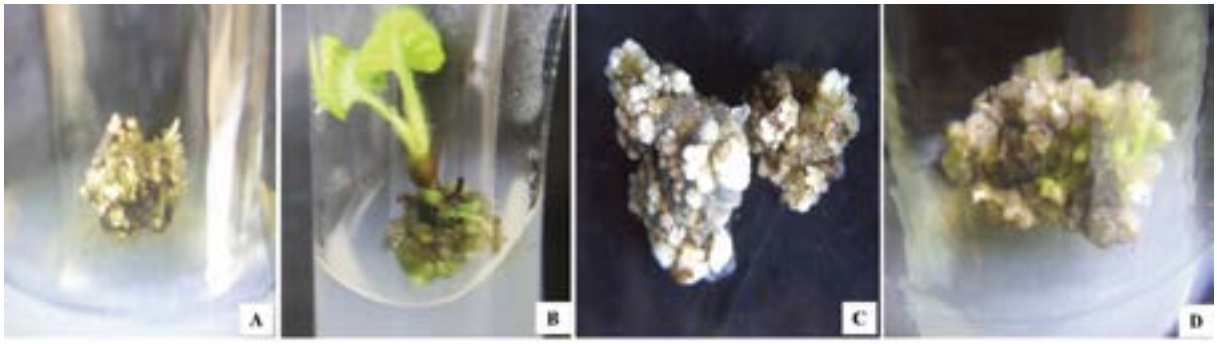

Figure 2: Embryogenic and nodular calli in *P. longum*: (A) Embryogenic calli from leaf explant; (B) Embryogenic calli from nodal explant; (C) Nodular calli from node explant at dark; and (D) Nodular calli from leaf explant at light.

The nodular aggregate calli from the leaf explants were induced on MS+1.5–4.5 mg/L 2, 4-D+10% CW alone, and in combination with 1.0–3.5 mg/L 2, 4-D+0.25–1.0 mg/L KN + 10% coconut water (Table 1 & 2). In the first subculture on MS + 3.5 mg/L 2, 4-D + 10% CW, the maximum frequency of embryogenic callus formation (50%) and the number of embryos (16.66±2.08) per 0.2–0.3 g fresh weight embryogenic callus was obtained.

Table 1: Frequency of embryogenic/nodular callus and somatic embryo (SE) development from leaf segment in the MS + 2, 4-D + 10% CW and control (MS only)

Explants	Media composition/Treatment	Embryogenic callus (%)	No. of countable SE/callus ± SD
Leaf segment	MS	-	-
Leaf segment	MS + 0.25 mg/L 2,4-D + 10% CW	-	-
Leaf segment	MS + 0.5 mg/L 2,4-D + 10% CW	-	-
Leaf segment	MS + 1.0 mg/L 2,4-D + 10% CW	-	-
Leaf segment	MS + 1.5 mg/L 2,4-D + 10% CW	16.6	4.00±1.00
Leaf segment	MS + 2.5 mg/L 2,4-D + 10% CW	33.3	13.66±4.50
Leaf segment	MS + 3.5 mg/L 2,4-D + 10% CW	50.0	16.66±2.08
Leaf segment	MS + 4.5 mg/L 2,4-D + 10% CW	33.3	6.33±1.52

In vitro condition: at 25±2° C temperature for 16 hours with 3000–4000 lux cool-white light intensity.

Similarly, in the first subculture on MS + 1.5 mg/L 2, 4-D + 1.0 mg/L KN + 10% CW, the maximum frequency of embryogenic callus formation (66.66%) and the number of embryos (28.33±3.511) per 0.2–0.3 g fresh weight embryogenic callus was obtained from the leaf segments (Table 2).

Table 2: Frequency of embryogenic/nodular callus and somatic embryo (SE) development from leaf segment in the MS + 2, 4-D + KN + 10% CW and in control (MS + 2, 4-D + KN)

Explants	Media composition/Treatment	Embryogenic callus (%)	No. of countable SE/callus ± SD
Leaf segment	MS + 0.25 mg/L 2, 4-D + 0.25 mg/L KN	-	-
Leaf segment	MS + 0.25 mg/L 2, 4-D + 0.25 mg/L KN + 10% CW	-	-
Leaf segment	MS + 0.5 mg/L 2, 4-D + 0.25 mg/L KN + 10% CW	-	-
Leaf segment	MS + 1.0 mg/L 2, 4-D + 0.25 mg/L KN + 10% CW	33.33	14.33±1.52
Leaf segment	MS + 1.5 mg/L 2, 4-D + 0.25 mg/L KN + 10% CW	50.00	16.33±2.51
Leaf segment	MS + 2.5 mg/L 2, 4-D + 0.25 mg/L KN + 10% CW	33.33	8.66±1.52
Leaf segment	MS + 3.5 mg/L 2, 4-D + 0.25 mg/L KN + 10% CW	16.66	7.00±2.00
Leaf segment	MS + 0.25 mg/L 2, 4-D + 1.0 mg/L KN + 10% CW	-	-
Leaf segment	MS + 0.5 mg/L 2, 4-D + 1.0 mg/L KN + 10% CW	-	-
Leaf segment	MS + 1.0 mg/L 2, 4-D + 1.0 mg/L KN + 10% CW	16.66	10.00±2.64
Leaf segment	MS + 1.5 mg/L 2, 4-D + 1.0 mg/L KN + 10% CW	66.66	28.33±3.05
Leaf segment	MS + 2.5 mg/L 2, 4-D + 1.0 mg/L KN + 10% CW	50.00	19.66±2.08
Leaf segment	MS + 3.5 mg/L 2, 4-D + 1.0 mg/L KN + 10% CW	33.33	12.33±2.51

In vitro condition: at 25±2° C temperature for 16 hours with 3000–4000 lux cool-white light intensity.

Moreover, the nodular aggregate calli from the stem explants were induced on MS media fortified with 0.5–4.5 mg/L NAA with 10% CW (Table. 3). However, the nodular callus did not induce on MS media supplemented with 2, 4-D alone and in combination with 2, 4-D and KN. The maximum frequency of embryogenic callus formation (50%) and the number of embryos (12.66±2.51) per 0.2–0.3 g fresh weight embryogenic callus was obtained in the first subculture on a medium supplement with 1.0 mg/L NAA.

Table 3. Frequency of embryogenic/nodular callus and somatic embryo (SE) development from the nodal segment in the MS + NAA + 10% CW and in control (MS only)

Explants	Media composition/Treatment	Embryogenic callus (%)	No. of countable SE/ callus ± SD
Nodal segment	MS	-	-
Nodal segment	MS + 0.25 mg/L NAA + 10% CW	-	-
Nodal segment	MS + 0.5 mg/L NAA + 10% CW	16.66	6.66±2.51
Nodal segment	MS + 1.0 mg/L NAA + 10% CW	50.00	12.66±2.51
Nodal segment	MS + 1.5 mg/L NAA + 10% CW	16.66	8.00±3.00
Nodal segment	MS + 2.5 mg/L NAA + 10% CW	33.33	9.00±2.00
Nodal segment	MS + 3.5 mg/L NAA + 10% CW	33.33	10.33±0.57
Nodal segment	MS + 4.5 mg/L NAA + 10% CW	16.66	4.33±1.52

In vitro condition: at 25±2° C temperature for 16 hours with 3000–4000 lux cool-white light intensity.

Somatic embryo development, maturation, and conversion into plantlets

The nodular callus developed into somatic embryos through 2-celled, 4-celled, 8-celled, globular, and torpedo stages on further subculture in the MS media fortified with 2, 4-D alone, NAA alone, and in combination with 2, 4-D + KN + 10% CW (Figures 3A–3I). Morphogenic differentiation of somatic embryos (whole plantlets/seedlings or shoots only), maturation, and conversion into seedlings were obtained when the nodular/embryogenic calli with somatic embryos, induced in the leaf explants, were transferred to the MS + 0.25–2.5 mg/L thidiazuron (TDZ) + 10% CW while those induced in the nodal explants were transferred to the MS + 0.25–3.5 mg/L TDZ + 10% CW (Table 4). However, seedlings were also differentiated from the nodular calli with somatic embryos in the MS media in the absence of PGRs.

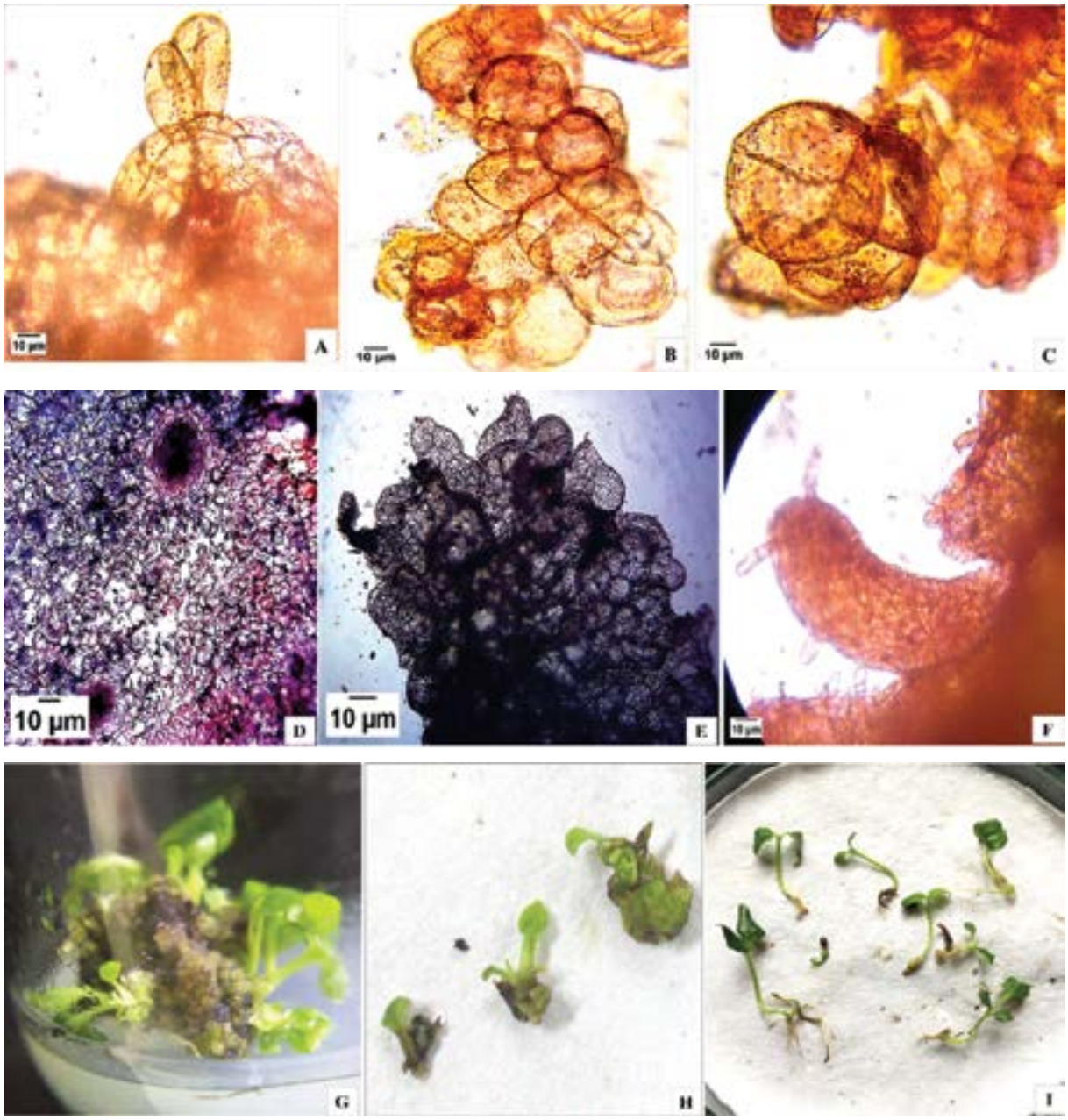

Figure 3: Various stages of somatic embryo development: (A) Two-celled stage; (B) Four-celled stage; (C) Eight-celled stage; (D) Globular stage; (E) Globular & torpedo stages; (F) Torpedo stages; (G) Multiple shoots from nodular callus; and (H & I) Mature isolated seedlings with roots.

In the case of the nodular callus induced from the leaf segment, the maximum frequency of the shoot differentiation (83.33%) and the whole plantlet (16.67%) differentiation occurred from the various stages of embryos in the MS + 0.5 mg/L TDZ + 10% CW, and was not found to be differentiated only into the roots and callus in any treatments (Table 4). However, in the case of the nodular callus induced from the nodal segment, the maximum frequency of the entire plantlet differentiation (66.66%) and the differentiation of the shoots alone (33.34%) occurred from the various stages of embryos, which was present in the nodular callus, in MS + 2.5 mg/L TDZ + 10% CW and was not found to be differentiated into roots only in any treatments, but in the MS + 0.25-0.5 mg/L TDZ + 10% CW, it induced callus only (Table. 4). However, in the case of the nodular callus induced from the nodal segment, the maximum frequency of the entire plantlet/seedling differentiation (66.66%) and the differentiation of shoots only (33.34%) occurred from the various stages of the embryos in the MS + 2.5 mg/L TDZ + 10% CW while differentiation into roots only were not noticed in in any treatments. The nodular callus induced from the nodal segments were not differentiated into whole plantlets and shoots only in the MS + 0.25–0.5 mg/L TDZ + 10% CW but produced callus only in this treatment. (Table. 4).

Table 4. Frequency of morphogenic response from leaf and nodal segments in the MS medium supplemented with TDZ

Explants/ Nodular calli	Media composition/ Treatment	Morphogenic response (%)			
		Shoots only	Roots only	Callus only	Whole plantlets
Leaf segment	MS	-	-	-	-
Leaf segment	MS+0.25 mg/L TDZ+ 10% CW	66.66	-	-	8.33
Leaf segment	MS+0.5 mg/L TDZ+ 10% CW	83.33	-	-	16.67
Leaf segment	MS+1.5 mg/L TDZ+ 10% CW	33.33	-	-	-
Leaf segment	MS+2.5 mg/L TDZ+ 10% CW	16.66	-	-	-
Nodal segment	MS	-	-	-	-
Nodal segment	MS+0.25 mg/L TDZ+ 10% CW	-	-	66.66	-
Nodal segment	MS+0.5 mg/L TDZ+ 10% CW	-	-	33.33	-
Nodal segment	MS+1.5 mg/L TDZ+ 10% CW	-	-	-	16.66
Nodal segment	MS+2.5 mg/L TDZ+ 10% CW	33.34	-	-	66.66
Nodal explant	MS+3.5 mg/L TDZ+ 10% CW	20.66	-	-	50.00

In vitro condition: at 25±2° C temperature for 16 hours with 3000–4000 lux cool-white light intensity.

Moreover, roots were regenerated from the shoots that were differentiated from the nodular calli regenerated from leaf segments in MS + 0.25-2.5 mg/L TDZ and nodal segments in MS + 2.5-3.5 mg/L TDZ when transferred to the MS media in the absence of PGRs (see Figure 4A). Similarly, some nodular calli containing somatic embryos were also differentiated directly into seedlings after being transferred

to the growth regulator-free full-strength MS media. For the acclimatization process, seedlings were grown in various substrates (Figure 4B & 4C).

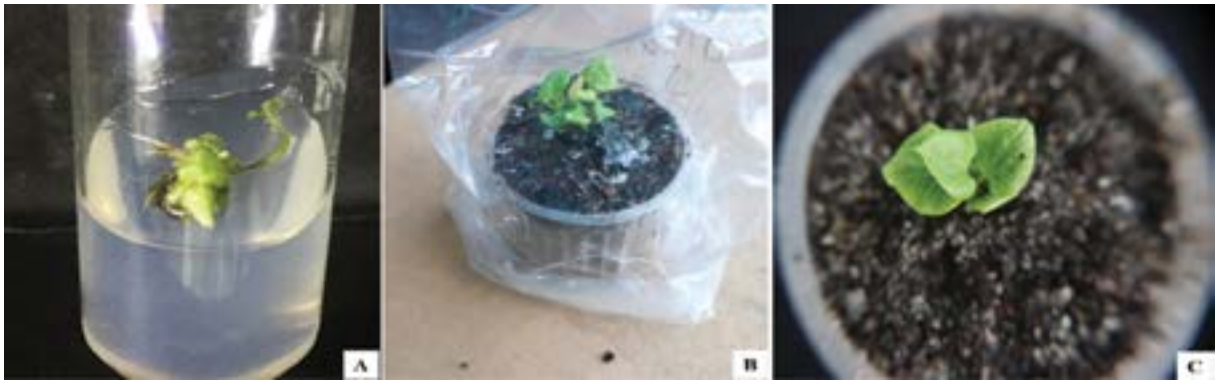

Figure 4: Embryo conversion: (A) Rooting seedling; and (B) & (C) Process of acclimatization of plantlets.

Discussion

P. longum is a valuable medicinal plant; however, sexual reproduction by seed production is difficult due to a lack of viable seeds and a shorter seed germination period (Sarasan *et al.*, 1993). Micropropagation by nodal explant culture, on the other hand, is difficult due to frequent contamination in culture by systemic endogenous bacteria (Bhat *et al*., 1995; Sathelly *et al*., 2016). As a result, *in vitro* propagation by somatic embryogenesis may be crucial for *ex-situ* conservation of *P. longum*. According to Williams & Maheswaran (1986), indirect embryogenesis generates somatic embryos through the development of callus. The induction of embryogenic callus from tissue/explant is the first stage in the formation of a somatic embryo. The embryogenic callus may change into a nodular callus at a later stage of growth, which is an embryogenic callus with compact and distinct spherical structures or nodules within a callus mass (Ferreira *et al.*, 2022). Embryogenic and nodular callus were not induced in the absence of 2, 4-D alone, NAA alone, and in the combinations of 2, 4-D and KN, with 10% CW in the MS media. However, the maximum percentage of embryogenic callus (66.66%) and somatic embryos (28.33±3.05) were induced from the leaf explants in the combination of the MS + 1.5 mg/L 2, 4-D +1.0 mg/L KN + 10% CW than that in the MS +3.5 mg/L 2, 4-D alone. It may be because the combination of 2, 4-D and KN has a synergistic effect on somatic embryogenesis by stimulating cell proliferation, differentiation, and the development of appropriate hormonal balance. The combined use of 2, 4-D and KN has been widely utilized to promote somatic embryogenesis in a variety of plant species (Zhang *et al.*, 2004; Joshee *et al.*, 2007; Raja *et al.*, 2012; Mazri *et al.*, 2017). Other studies showed that somatic embryos were developed in the MS media in combination with KN and 2, 4-D in some plants such as *Centella asiatica* (Joshee *et al.*, 2007), *Epipremnum aureum* (Zhang *et al.*, 2004), and *Phoenix dactylifera* (Mazri *et al.*, 2017). Similarly, the 2, 4-D is an auxin that induces somatic embryogenesis, but auxins may require another auxin or cytokinin in association to induce somatic embryos (Yong-Wook, 2000). A lower concentration of 2, 4-D alone as well as in combination with KN did not induce nodular callus and somatic embryos in this study. Therefore, this study showed that induction of nodular callus and somatic embryos in the leaf segment is preferred by a higher dosage of 2, 4-D & a lower dosage of KN in MS media. Some researchers also developed somatic embryos from leaf segments in other combinations of PGRs, such as MS + BA + KN in *P. colubrinum* (Yusuf *et al.*, 2001) and MS + NAA + BAP in *P. aduncum* (De Sousa *et al.*, 2020), demonstrating that effective somatic embryos can develop in combination of both cytokinins as well as auxins and cytokinin.

Moreover, the maximum frequency of embryogenic callus induction (50%) and the number of somatic embryos (12.66±2.51) were obtained in the MS medium fortified with 1.0 mg/L NAA alone and 10% coconut water in the callus induced from the nodal explants of *P. longum*. These results were supported by the findings of Xu *et al.* (2019) in *Ranunculus scleratus,* where somatic embryos were induced from the stem, leaf, and root at higher concentrations of NAA. The results were equally supported by the findings of Pinto *et al.* (2002), where somatic embryos were induced from the callus regenerated from cotyledons & from whole mature zygotic embryo explants at higher concentration of NAA in *Eucalyptus globulus*. The results were also supported by the findings of Zdravkovic-Korac *et al.* (2023), where somatic embryos were induced from the roots of spinach (*Spinacia oleracea* L.) in the MS + 20 µM NAA + 5 µM GA_3. Mazri *et al.* (2017) and Szewczyk-Taranek & Pawlowska (2015) also developed somatic embryos in *Phoenix dactylifera* bud explants and Szewczyk-Taranek & Pawlowska (2015) produced somatic embryos from the embryogenic callus induced from seedlings of *Hepatica nobilis* at 1.0 µM concentration of NAA. NAA can stimulate somatic embryogenesis by boosting cell division and differentiation, activating embryogenic pathways, restoring hormonal balance, initiating dedifferentiation, and encouraging the organogenesis process (Bhatia, 2015). However, Venkatachalam *et al*. (1999) found the 2, 4-D to be a more effective auxin for the induction and production of somatic embryos as compared to NAA.

Another crucial step during somatic embryogenesis is the differentiation, development, and transformation of somatic embryos into seedlings/plantlets from the various stages of embryos present in the nodular callus. When the nodular callus formed from both the leaf and nodal explants was further sub-cultured in the same composition of media, it differentiated into somatic embryos ranging from 2-celled to torpedo stage embryos (see Figure 2), but embryo differentiation into multiple shoots or whole plantlets did not occur in the absence of TDZ and 10% CW. Nhut *et al.* (2006) observed that TDZ, whether alone or together with other plant growth regulators, can stimulate somatic embryogenesis in a tissue culture medium. Altering the auxin to cytokinin proportion *in vitro* results in somatic embryogenesis in somatic cells (Murthy *et al.,* 1998), while TDZ alone can also cause somatic embryogenesis in many species (Murthy *et al.,* 1998; Ghosh *et al*., 2018; Erland *et al.*, 2020). Sreenivasu *et al.* (1998) used TDZ alone to stimulate somatic embryogenesis in *Azadirachta indica* and *Cajanus cajan*. Moreover, coconut water might act as a natural and useful ingredient in tissue culture media for somatic embryo differentiation, supplying important nutrients, growth hormones, osmotic management, and antioxidant protection to aid in the growth and development of embryos. The maximum percentage of somatic embryo conversion (83.33% shoots only and 16.67% whole plantlets) was found in the MS + 0.5 mg/L TDZ + 10% CW from nodular callus induced from the leaf explants, while the maximum percentage of somatic embryo conversion (33.33% shoots only and 66.66% whole plantlets) was found in the MS + 2.5 mg/L TDZ + 10% CW from the nodular callus induced from the nodal explants. It demonstrates that a lower TDZ concentration (0.25 to 0.5 mg/L) supports the differentiation of embryos into shoots only whereas a higher TDZ concentration (2.5 mg/L) favors the conversion into seedlings in *P. longum* from the various stages of embryos that were present in nodular callus. Thus, the percentage of whole plantlet conversion was higher in the nodular callus developed from the nodal explants than in the nodular callus developed from the leaf explants. It may be due to the residual effect of NAA in somatic embryos that was induced from nodal explants for *in vitro* rooting. On the other hand, after embryo differentiation, all the embryos developed into seedlings when they were grown in the full-strength MS media in the absence of PGRs. This observation has been reinforced by the findings of Mazri *et al.* (2017) in *Phoenix dactylifera*. However, Simos *et al*. (2010) found that not all embryos were transformed into seedlings (plantlets), but a small percentage were converted into roots only and callus only in the MS media in the absence of PGRs. In some *Piper* species, including *P. nigrum*, somatic embryogenesis

was reported from micropylar tissues or zygotic embryos in Schenk & Hildebrandt (SH) media in the absence of hormones at dark (Nair & Gupta, 2003, 2006; Sasi & Bhat, 2016).

Conclusion

The efficient protocol for the development of somatic embryos in *P. longum* was established using the leaf and nodal explants in the MS media fortified with 2, 4-D alone, NAA alone, & when used together with 2, 4-D, KN, and 10% coconut water. The efficacy of nodular calli induction in the case of the leaf segment was higher in the MS + 2, 4-D + KN than in the MS + 2, 4-D alone. The nodular callus with embryos, which was induced from both the leaf and nodal explants, was differentiated into entire plantlets/seedlings and shoots only in the MS media fortified with TDZ and 10% coconut water. The matured embryo conversion into seedlings also took place without the addition of growth regulators in the full-strength MS media. This research could aid in the *ex-situ* conservation of *P. longum* by providing an alternative approach for its successful micropropagation.

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Author's contribution statement

C. B. Thapa: Conceptualization of study, data curation, data analysis, and original draft writing. K. K. Pant: Formal data analysis, review, and editing. H. D. Bhattarai: Data analysis, review, and editing. B. Pant: Conceptualization of the study, supervision, review, and final editing.

Data availability

The raw data utilized in this study can be obtained on request from the corresponding author.

Conflict of interest

The authors have no conflicts of interest in this study.

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