Unlocking Regeneration Potential: The Role of Phytosulfokine-α in Foxtail Millet Protoplast Systems

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Abstract

Foxtail millet (Setaria italica L.) is one of the most drought-tolerant crops, and is well- suited to cultivation in stress-prone environments. In the present study, we report the development of an efficient regeneration protocol using protoplasts derived from stem and sheath leaves, along with the establishment of a transient transformation system. The 14-day-old in vitro-grown seedlings of foxtail millet were digested in an enzymatic solution containing 1.0% (w/v) cellulase, 0.4% (w/v) macerozyme R-10, and 0.6M mannitol in the dark for 4 hours. This treatment resulted in the highest protoplast yield (2.9 × 10^6/g·FW) and viability (84.67%). Protoplasts, at a plating density of 4×105 protoplasts/mL, were embedded in a thin alginate matrix (TAL) and cultured in protoplast culture medium (PCM-I & PCM-II) supplemented with 0.1 µM phytosulfokine-α (PSK). This approach facilitated the protoplasts to divide and achieve the highest plating efficiency of 37% on day 47. The microcalli were then released from the alginate layer and proliferated continuously on callus culture medium (CCM) supplemented with 0.1µM of PSK. Protoplast-derived calli were successfully regenerated into plantlets after 6-7 months, with a shoot regeneration frequency of 10.3%. Furthermore, the transient transformation of protoplasts was optimized using 30% PEG4000 and 30 µg of plasmid DNA, resulting in a transformation efficiency of 79.3%. These findings contribute to the development of efficient protocols that will support future functional genomic studies and crop improvement efforts in foxtail millet.

Introduction

Foxtail millet (*Setaria italica* L.) is renowned to be the one of the most drought-tolerant crops and is well adapted to grow in variety of stressful environments, particularly across the Asia and Africa. This plant serves as a valuable research model due to its notable characteristics, which include a small genome size, a short developmental life cycle, and close genetic relatedness to several cereals and millets[1-2]. It is being extensively used for studying C4 photosynthesis[3], abiotic stress biology[4], and biofuel trait research[5]. Although foxtail millet is acknowledged as a promising candidate for advancing research in millet biotechnology, the development of genomic tools to support its genetic improvement has progressed significantly much more slowly compared to other gramineous crops like rice, maize, and barley[6]. To meet the growing global demand for food and feed, continuous advancements in cultivar development are crucial for breeders and producers[7]. Inter- and intra-specific hybridization within the Poaceae family provides valuable genetic resources, enabling the introduction of novel traits and improving crop performance[8-9]. Establishing a reliable and reproducible protoplast-based regeneration protocol is fundamental to fully realize its potential for genetic improvement and biotechnological applications[10].

The improvement of plant varieties with desirable traits using protoplast technology is gaining significance, particularly through somatic hybridization and transformation techniques in species that are difficult to regenerate[11]. However, establishing a reliable and efficient protocol for protoplast isolation, culture, and regeneration, is a key step towards further advancing plant genetic manipulation

and incorporating it into breeding programs[12]. Studies conducted previously have shown that efficient methods for protoplast isolation are highly effective in certain cereals crops, including wheat[13], maize[14], rice[15], sorghum[16], proso millet[17] and perennial ryegrass[18]. Numerous fundamental studies have offered important insights into the key processes involved in protoplast regeneration, including the cell-wall reconstruction, callus induction, and de-novo tissue regeneration, which differ notably from the *in vitro* plant regeneration derived from tissue explants[10, 19-20]. The conventional liquid culture approach for protoplast regeneration offers a relatively simple and straightforward manner of inducing cell division and callus formation. However, this method is often encountered with low proliferation efficiency, primarily due to cell aggregation-induced cell death[21]. Protoplast-embedding techniques using low-melting agarose or alginate have been developed to immobilize protoplasts through crosslinking with calcium (Ca2+) ions, thereby overcoming the limitations encountered in liquid culture[22]. This approach has been successfully applied in various plant species, including *Petunia hybrida*[23], *Nigella damascene*[24], *Arabidopsis thaliana*[25], *Solanum tuberosum*[26], *Camellia oleifera*[27], *Brassica oleracea*[28], *Pastinaca sativa*[29], and *Solanum lycopersicum* [30]. However, very few studies have been reported on protoplast culture in foxtail millet using suspension culture[31].

The addition of further supplements to protoplast cultures can enhance the cell viability and stimulate cell division, thereby facilitating callus formation and regeneration. A notable example is phytosulfokine-α (PSK-α), a sulphated pentapeptide that known to promote cell growth and proliferation[32]. Previous studies on PSK-α have shown its effectiveness in promoting callus[33], shoot[34], and root formation[35], as well as in improving somatic embryogenesis[36]. The influence of PSK-α on *in vitro* protoplast proliferation has been investigated in several plant species, including *Beta vulgaris*[37], *Brassica oleracea* var. *capitata*[38],*Daucus carota*[39] and *Fagopyrum tataricum*[40]. In recent years, transient protoplast transformation systems have served as a rapid and efficient alternative to long-term stable expression methods for evaluating a large number of functional genes, protein-protein interactions and molecular signaling processes within a significantly shorter timeframe[41]. Protoplast transient expression studies are now playing a progressive role in the development of plant CRISPR/Cas-based editing tools for validating guide RNA efficiency and to assess editing outcomes prior to stable transformation[42].
The introduction of foreign DNA using polyethylene glycol (PEG) for transient expression is a simple, cost-effective technique that offers significant advantages in enhancing transformation efficiency and reproducibility[43]. In recent years, successful protocols for protoplast-based transient expression have been developed for numerous plant species, including *Camellia sinensis*[44], *Brassica oleracea* L. var. *italica*[45], *Uncaria rhynchophylla*[46].

In this study, we describe an efficient protocol for protoplast isolation and examine the positive influence of PSK-α on the viability and division frequencies of foxtail milletprotoplasts embedded within calcium alginate layers. Furthermore, the efficient PEG-mediated transient transformation system established here provides a simple and reliable platform for further protein molecular interaction studies and other *in vivo* assays.

Results

Protoplast yield and viability screening

After four hours of incubation, spherical protoplasts derived from the stems and sheaths of foxtail millet seedlings were successfully released into the enzyme solution (Fig. 1a). The mean yield of protoplasts varied with enzyme concentration, ranging from 1.5×10^6 to 2.9×10^6 (Table 1), with an average yield of 2.33×10^6 protoplasts per gram of fresh weight (FW). Different cellulase concentrations exhibited varying enzymatic activities, affecting both the yield and viability of the protoplasts (Fig. 1b). The highest yield was obtained with a 1.0% enzyme solution ($2.9 \pm 0.69 \times 10^6$), while increasing the enzyme concentration to 2% reduced the number of released protoplasts by approximately half. The concentration of PEG solution is a key factor influencing the stress response of protoplast cells during transfection. In the transfection optimization experiment, protoplasts exposed to 40% PEG exhibited the lowest viability, averaging 61.23%. Comparable results were obtained with 10% (63.77%) and 20% (65.62%) PEG solutions (Fig. 1c). In contrast, the 30% PEG treatment produced the highest viability at 69.26% following incubation, indicating that this concentration is optimal for transformation while minimizing protoplast loss. Protoplast quality was evaluated immediately through fluorescein diacetate (FDA) staining, which indicated significant variations in viability across different enzyme concentrations (Table 1). Viable protoplasts retained a smooth, spherical morphology without noticeable contraction or shrinkage. The greatest viability, reaching 84%, was observed in samples treated with a 1.0% cellulase solution, while increasing the concentration to 2.0% reduced viability to 69%.

Protoplast division and microcallus formation

Foxtail millet protoplasts exhibited a progressive response to alginate immobilization and optimized culture media (PCM-Ia and PCM-IIa), which improved their developmental competence. Supplementation of these media with phytosulfokine (PSK) further stimulated protoplast division, resulting in the formation of multicellular aggregates (Fig. 2f). Cell division rates were similar in PCM-Ia and PCM-IIa media (data not shown). Protoplasts cultured in the above media exhibited an early onset of mitotic activity, with the first visible cell divisions occurring around the 10th day of culture (Fig. 2g). Following this initial phase, both cell division and the formation of microcolonies progressed steadily over the subsequent weeks. In contrast, cultures without PSK in PCM-I and PCM-II media acted as controls. In these control cultures, minimal to no cell division was observed until approximately day 20, at which point only about 2.5% of protoplasts had initiated division, forming a few small aggregates. By day 47, these aggregates gradually increased to about 21.5% (Fig. 2h; Fig. 2i, j). The proportion of dividing cells and newly formed colonies in the presence of PSK reached between 21% and 46% by day 26 (Fig. 2k, l) and further increased to between 25% and 37% by day 47 (Fig. 2m, n). The mean plating efficiency was evaluated, and the highest efficiency was observed in PSK-supplemented medium, showing 25.6% aggregate formation on day 33, which increased to 37.3% by day 47 (Fig. 2o, p). These values were notably higher than those in cultures treated with a lower PSK concentration (0.01 μM), which produced 21.8% aggregates on day 33 and 34.6% by day 47. In contrast, cultures supplemented with a high PSK

concentration (1.0 μM) showed adverse effects, resulting in a marked decrease in cell division and aggregate formation. The proportion of aggregates dropped to 12.8% by day 26 and further declined to 7.53% by day 47 (Table 2).

Shoot regeneration from protoplast-derived callus

After approximately two-to three-months of culture, distinct protoplast-derived calli became visible to the naked eye, marking a critical stage in the regeneration process. These developing calli were carefully relaesed from the surrounding alginate matrix. The resultant calli were transferred to fresh callus multiplication medium (CCM) to promote further growth and development. Over the course of three to four weeks of incubation, the calli gradually increased in size, reaching an average diameter between 0.5 and 0.8 centimeters (Fig. 2q). During this phase, the tissue exhibited active proliferation and a compact, friable texture typical of healthy callus growth. Continued subculturing on CCM led to enhanced cellular organization, ultimately resulting in the formation of a dense, homogeneous pro-embryogenic mass (Figs. 2r, s). The highest number of callus clumps, 38.5 ± 0.18 per Petri dish, was observed with 0.1 μM PSK. The regeneration capability was higher in callus colonies cultured in SRM supplemented with PSK, with a regeneration frequency of 10.3 ± 0.76% (Fig. 2t, u) (Table 3). When transplanted onto SRM without PSK, the calli failed to proliferate effectively, showing only 3.6% of shoot proliferation, and exhibited signs of browning and desiccation. Supplementation with higher concentrations of PSK, resulted in fewer calli clumps, with only 6.3% per plate, and a reduced shoot regeneration frequency of 1.2% (Table 3). After one month of culture, the plants successfully developed roots on the rooting medium (RM) (Fig. 2v, w). The entire protoplast-to-plant regeneration process in *Setaria italica* L., took around 6–7 months to produce plantlets.

Protoplast transient transformation efficiency

Based on the outcomes of the transformation stimulation experiment, it was determined that polyethylene glycol (PEG) with a molecular weight of 4000 at a concentration of 30% (w/v) provided the most effective conditions for transient gene expression using the PCaM35S promoter-driven GFP reporter construct (Fig. 3a, b). This concentration of PEG4000 facilitated efficient uptake of plasmid DNA into foxtail millet protoplasts, while maintaining cell integrity and minimizing cytotoxic effects. To further refine the transient transformation protocol, the influence of incubation time and plasmid DNA amount on transfection efficiency was systematically examined. When the incubation period during PEG-mediated transfection was increased from 10 to 30 minutes, the proportion of GFP-expressing protoplasts rose markedly from 20.7% to 71.9%. This sharp increase indicates that longer exposure enhances membrane permeability and promotes more effective DNA uptake. However, when the incubation time exceeded 30 minutes, a noticeable decline in transformation efficiency was observed. This reduction likely resulted from prolonged PEG exposure, which can lead to osmotic stress and damage to the protoplast membrane. Therefore, an incubation duration of approximately 30 minutes was identified as the optimal balance between maximizing gene delivery and maintaining protoplast

viability for transient expression assays in foxtail millet (Fig. 4a). The effect of varying plasmid concentrations on transformation efficiency was evaluated using 250µL of protoplasts resuspended in W5 solution. At a plasmid concentration of 10µg, the transformation efficiency was 21.37%, which increased to 55.6% and 75.3% with 20µg and 30µg of plasmid, respectively (Fig. 4b). However, a further increase to 40µg resulted in a marked decline in efficiency to 43.7%.

Discussion

Compared to major cereals like rice, barley, wheat and maize, millets have received limited research attention, leading to slow progress and a lack of standardized protocols for regeneration and transformation. Active efforts are currently ongoing to address these challenges in foxtail millet and paving the path for achieving significant advancements in functional genomics and genome editing techniques to improve the crop traits[47]. In this study, we aimed to establish an efficient protoplast isolation and culture method for *Setaria italica* L., the most widely planted species of millet in Asia. Despite its importance as an annual food grass, research focused on the utilization of *in vitro* cultures in this species is limited[48]. Given these gaps in the literature, the present work seeks to optimize protoplast isolation and culture conditions to facilitate future genetic, physiological, and molecular studies in this important crop species. Only a limited number of studies have addressed the isolation and cultivation of foxtail millet protoplasts, with most previous efforts relying primarily on cell suspension cultures.[31,49]

Choice of donor tissue, enzyme concentration, incubation duration, culture conditions, and growth medium composition are the key factors that determine the success of protoplast studies.[50] Younger plant tissues, particularly mesophyll cells, typically yield higher-quality and more viable protoplasts[51]. In this study, green stem and sheath tissues from 14-day-old *in vitro* grown seedlings were selected as the protoplast source. Comparable approaches have been reported in other members of the Poaceae family[15]. Using this tissue source for protoplast isolation offers advantages over traditional suspension cultures by minimizing somaclonal variation and reducing the cost associated with long-term culture systems[52].

Balancing the duration of enzymatic hydrolysis with enzyme concentration is crucial for maximizing protoplast yield and viability[53]. In this study, protoplasts were isolated using enzymatic hydrolysis with 1.0% cellulase and 0.4% macerozyme, followed by a four-hour incubation in darkness. Increasing the cellulase concentration to 1.5% or 2.0% led to a significant reduction in both protoplast yield and viability. These findings are consistent with previous research, which similarly highlights the importance of optimizing enzymatic conditions. Studies on *Arabidopsis thaliana*[54], *Populus euphratica* Oliv.[55], *Brassica rapa*[56], and *Camellia oleifera*[57] have shown that excessive enzyme concentrations or prolonged digestion times negatively affect cell viability. Although sufficient digestion is necessary for efficient cell wall breakdown, overly long incubation can cause cellular damage and reduce viability[58]. The optimal duration of enzymolysis also varies depending on the tissue type used for protoplast isolation. For instance, in *Ricinus communis*, protoplasts derived from callus tissue required approximately seven

hours of digestion[59], whereas those obtained from cotyledons needed about nine hours[60]. The successful division of protoplasts and their subsequent regeneration into complete plantlets are critically influenced by the composition of the culture medium and the cultivation protocols employed. Media based on Murashige and Skoog[61] or Gamborg's B5[62] are commonly used, although their optimal formulations vary with species, genotype, and tissue source, suggesting that multiple formulations can effectively promote early protoplast development under optimized conditions. The balance between cytokinins and auxins is critical, as it governs cell division, dedifferentiation, and morphogenesis[10]. In this study, two media (PCM-I and PCM-II) were tested with different nutrient and hormone concentrations. Both supported similar levels of cell division, indicating that multiple formulations can effectively promote early protoplast development under optimized conditions.

Osmotic pressure plays a crucial role in the success of protoplast-based culture systems, as it directly influences the stability and viability of isolated protoplasts. Commonly used agents include mannitol, glucose, sucrose, and myo-inositol, each of which helps to regulate the external osmotic environment[50]. In this protocol, mannitol and glucose served as the main osmotic regulators, creating favorable conditions for foxtail millet thereby ensuring optimal conditions for protoplast survival, cell wall regeneration, and subsequent cell division and growth. Along with osmoticum, plating density also influenced microcolony viability; a density of 4×10^5 protoplasts/ml significantly enhanced the formation of viable microcolonies. Comparable results have been observed in petunia cultivars, where similar densities promoted cell division and microcallus formation[63]. The technique used for embedding protoplasts represents another critical factor that can profoundly affect the success of protoplast culture and regeneration. Various embedding strategies have been developed to provide a supportive microenvironment for isolated cells. These include suspending protoplasts in thin layers or droplets of agar or agarose, employing feeder layers or nurse cultures, and placing filter paper overlays on agar media to facilitate nutrient and signal exchange[22]. Immobilizing protoplasts within a semi-solid matrix not only minimizes cell aggregation but also enhances stability, nutrient diffusion, and overall cell viability during the early stages of culture. This method has been shown to be effective for several species, including *Nigella damascena*[24], *Arabidopsis thaliana*[30], *Fagopyrum esculentum*[64], and recently *Brassica oleracea*[65]. Thin layers of alginate gels for foxtail millet protoplasts have a positive impact on cell division and colony formation.

Another approach to enhance protoplast division and microcallus formation involves the supplementation of culture media with specific additives, such as peptide growth factors (PSK), as these compounds are known to stimulate plant cell growth and development[66]. Our results showed that adding 0.1 μM PSK-α to the culture medium enhanced cell division and aggregate formation, whereas lower or higher concentrations (0.01 and 1.0 μM) decreased microcallus formation and organogenesis efficiency. Similar improvements in protoplast cell culture induced by PSK have been reported in *Daucus sps*[39], *Brassica oleracea*[38] and *Pastinaca sativa*[29]. Additionally, Hanai et al. (2000)[67] and Grzebelus et al. (2012)[37] demonstrated that PSK can overcome the recalcitrant nature of mesophyll protoplasts derived from *Daucus carota* and *Beta vulgaris*, respectively. Comparatively in our investigation, we found that

PSK-α supplementation had a beneficial impact on the regeneration process. For shoot regeneration, KM[51] medium, which is rich in minerals and organic matter, was employed. Callus colonies grown on medium supplemented with PSK-α produced a greater proportion of regenerated shoots (10.3 ± 0.76). PEG-mediated transient transformation, which employs polyethylene glycol (PEG) provides a reliable platform for assessing protein interactions, subcellular localization, and gene construct performance. However, its transfection efficiency varies considerably among plant species[15]. The transformation experiment demonstrated that a 30% PEG 4000 concentration achieved the highest transformation efficiency in protoplasts, while deviations from this optimal level led to a marked decline in efficiency. Similar observations were reported by Tan et al. (2013)[68] in *Populus* species. Additionally, the study examined the optimal plasmid concentration and incubation duration for transient transformation, parameters known to differ among plant species[69–70,27]. The results indicated that using 30 µg of plasmid DNA and a 30-minute incubation produced the highest transformation efficiency in foxtail millet protoplasts.

Materials and Methods

Plant material

Mature seeds of *Setaria italica* L. (German foxtail millet; commercially available) were first rinsed with running tap water for 5–10 minutes to remove dust and surface debris. The seeds were then transferred to a sterile beaker and immersed in 70% (*v*/*v*) ethanol for 1–2 minutes with gentle swirling, followed by three rinses with sterile water. This was immediately followed by treatment with 5% sodium hypochlorite solution containing 100 µL of Tween-20 per mL for 30 minutes, with intermittent shaking to ensure uniform exposure. After sterilization, the seeds were rinsed 3–4 times with sterile distilled water, air-dried, and cultured in autoclaved polypropylene vessels (80 mm × 60 mm) fitted with microporous filters. Approximately 100–150 seeds were sown per vessel on solid MS medium[61] supplemented with 30 g/L sucrose and 0.8% agar. Cultures were maintained at 24 ± 2°C under a 16-hour light period (55 µmol m − 2 s − 1). Green stem and sheath tissues from 10–14-day-old seedlings (approximately 60–100 plants) were used for protoplast isolation.

Extraction of protoplasts

To isolate protoplasts, bundles of aseptically grown millet seedlings were cut into 0.5 mm sections under sterile conditions and immersed in 10–15 mL of mannitol-based plasmolysis solution (PS) in a 100 mm Petri dish. The dish was covered with dark foil and placed on a gyratory shaker operating at 50 rpm for one hour. After incubation, the plasmolysis solution was removed and replaced with 15–20 mL of filter-sterilized enzyme solution (ES) (Fig. 2a, b) (Table 4). The tissue–enzyme mixture was vacuum-infiltrated at − 40 to − 50 kPa (0.4–0.7 bar) for 5–10 minutes to ensure enzyme penetration, then incubated at room temperature for four hours in the dark with gentle shaking (40–50 rpm). After sufficient digestion, the mixture was filtered through a 70 µm nylon mesh to remove debris, and the

filtrate was centrifuged in a swinging-bucket rotor at 100 × *g* for 5 minutes (acceleration @ 6; deceleration @ 1) at 4°C in a sterile 15 mL falcon tube. The resulting pellet was washed twice to remove residual enzymes by gently resuspending it in 5–10 mL of W5 salt solution, and finally resuspended in 2 mL of MMg solution. The suspension was carefully layered over 0.5 M sucrose solution in a 5 mL Eppendorf tube using a sterile disposable transfer pipette. Following centrifugation at 100 × *g* for 10 minutes, a distinct thick band containing intact protoplasts appeared at the interface. These protoplasts were carefully collected, washed twice, and resuspended in 1 mL of MMg solution (Fig. 2c, d).

Protoplasts yield and viability

Protoplast yield was determined after purification by loading 10 µL of the cell suspension onto Countess™ cell counting chamber slides (Invitrogen) and counting the cells. The working density of protoplasts was adjusted to 7.5×10^5 protoplasts per mL in MMg solution (Fig. 2e). To initially assess the impact of osmotic shock, a transfection simulation experiment was conducted without introducing plasmid DNA. Protoplasts were treated with varying concentrations of PEG 4000 solutions (10%, 20%, 30%, and 40% w/v), and viability was evaluated after 16 hours of incubation in W5 solution. Subsequently, protoplast viability was determined using fluorescein diacetate (FDA) staining[71]. For staining, 10 µL of FDA stock solution (5 mg per mL in acetone) was added to 1 mL of protoplast suspension to achieve a final concentration of approximately 0.01% (v/v). The suspension was mixed gently and incubated at room temperature for 5–10 minutes in the dark. Stained protoplasts were then examined using a ZEISS Axio Zoom.V16 fluorescence microscope equipped with a blue excitation filter (around 490 nm).

Protoplast embedding and culture

The purified protoplasts were immobilized using the Thin Alginate Layer (TAL) method, described by Grzebelus et al. (2012)[37] with minor modifications. The sodium alginate solution, sterilized by autoclaving at 121°C for 10 minutes and allowed to cool to room temperature, was gently mixed in a one-to-one ratio with the protoplast suspension, adjusting the final concentration to 4×10^5 protoplasts per mL. Approximately 800 µL of the protoplast–alginate mixture was evenly spread across the surface of pre-prepared $CaCl_2$-agar Petri dishes. The plates were gently rotated either in clock-wise or anti-clockwise direction on a flat surface to ensure an even and uniform layer thickness. The protoplast-alginate layers were then left to solidify for 45 minutes, allowing complete polymerization. The resulting thin alginate-protoplast layers were carefully transferred into 60 x 15mm Petri dishes using two sterile forceps and then add 4 ml of protoplast culture medium (PCM). In the initial experiments, the embedded protoplasts were cultured in two different liquid culture media, PCM-I and PCM-II (Table 5). Cultures were maintained at 25°C in the dark for 7–8 weeks, and the medium was refreshed every 20 days. In subsequent trials, the effect of the peptide hormone on protoplast division was examined by

supplementing the media (PCM-Ia and PCM-IIa) with phytosulfokine-α (PSK; PhytoTech Labs; P6633) at different concentrations (10, 100, or 1,000 nM) [29].

Alginate degradation and shoot regeneration from protoplast-derived callus

After 7–8 weeks of culture in protoplast culture media, the plates were subsequently incubated at 25°C under continuous dim light conditions, achieved by covering them with a double layer of cheesecloth, for 4–5 weeks to stimulate microcallus development. Finally, the protoplast culture medium was discarded, and 5–10 mL of sodium citrate solution, as described in Table 4, was added to dissolve the alginate layer. The Petri dish was kept at a slight angle for about 45 minutes until the alginate gel fully suspended and gets dissolved. The sodium citrate solution was then gently removed by pipetting. Callus production was quantified as the average number of callus clumps at least 0.5 mm in diameter formed per Petri dish. The protoplast-derived microcolonies (diameter > 1 mm) were rinsed twice with MMg solution, gently blotted on sterile filter paper, and transferred to callus culture medium (CCM) (Table 5). After 4–5 weeks of proliferation, the resulting green embryogenic callus was moved onto solid shoot regeneration medium (SRM) (Table 5) to induce de novo shoot formation. Regeneration frequency was calculated as the percentage of shoots regenerated in relation to the total number of calli maintained on the regeneration medium. After 5 to 6 weeks of incubation under continuous light at an intensity of 55 µmol m^{-2} s^{-1} and a temperature of 25°C, the regenerated shoots were excised and transferred to rooting medium (RM) as described in Table 5, where they were cultured for an additional 2 to 3 weeks to induce root formation[29].

Protoplast transient transformation

For transient expression of green fluorescent protein (GFP), protoplasts prepared using the previous procedure were suspended in 1 mL of MMg solution and adjusted to a concentration of approximately 1 × 10^6 cells per mL. The suspension was kept on ice for 30 minutes. Aliquots of 250 µL were then transferred into separate round-bottom tubes, and plasmid DNA was added at concentrations of 10, 20, and 30 µg. Each sample was mixed gently to ensure even contact between the DNA and the protoplasts. Transformation was performed using a plasmid carrying the *gfp* gene under the control of the CaMV 35S promoter. An equal volume of 30% PEG solution was introduced to each tube and mixed slowly to promote uniform interaction. The reaction mixtures were incubated at room temperature in the dark for 10, 20, or 30 minutes to determine the optimal exposure period. To terminate the transformation, twice the reaction volume of W5 solution was added. The cells were then centrifuged at 100 × *g* for 5 minutes, washed twice to remove residual PEG, and gently resuspended in fresh MMg solution. The resulting protoplasts were stored at 4°C overnight to allow recovery. GFP fluorescence was examined under a fluorescence microscope. Transformation efficiency was calculated as the proportion of GFP-positive cells relative to the total number of protoplasts observed in each field of view, using the formula: (GFP-positive cells / total cells) × 100. Observations were recorded from five to ten randomly selected microscopic fields to ensure accuracy.

Data collection and statistical analysis

Data were collected from three independent trials, each treatment replicated across four petri dishes. Protoplast yield was expressed as the number of protoplasts per gram of fresh tissue. Microscopic examination was performed to evaluate cell viability and plating efficiency, using counts from a minimum of five fields per petri dish, each containing 200–400 cells. Viability was calculated as the percentage of protoplasts showing green fluorescence out of the total number of cells observed (×100). Plating efficiency was assessed in 48-day-old cultures and calculated as the percentage of cell aggregates relative to the total number of undivided cells and cell colonies observed (×100). Data are presented as mean ± standard error (SE) from three independent experiments. Statistical analyses were performed using SPSS Version 18.0 (SPSS Inc., Chicago, IL, USA) with one-way ANOVA at a significance level of $P \leq 0.05$. Mean differences were evaluated using Tukey's HSD test.

Declarations

Data availability

All data is presented within this manuscript.

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Author contributions

M.K.N. Conceptualization, writing of the original draft. T.S., *B.P., and H.Y.* Experimentation, Writing and Reviewing. Z.R. Writing and Reviewing.P.B. Supervision, Writing, Review and Editing. All authors approved the final manuscript.

Competing Interest declaration

The authors declare no competing interests.

Additional information

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Tables

Tables 1 to 5 are available in the Supplementary Files section.

Figures

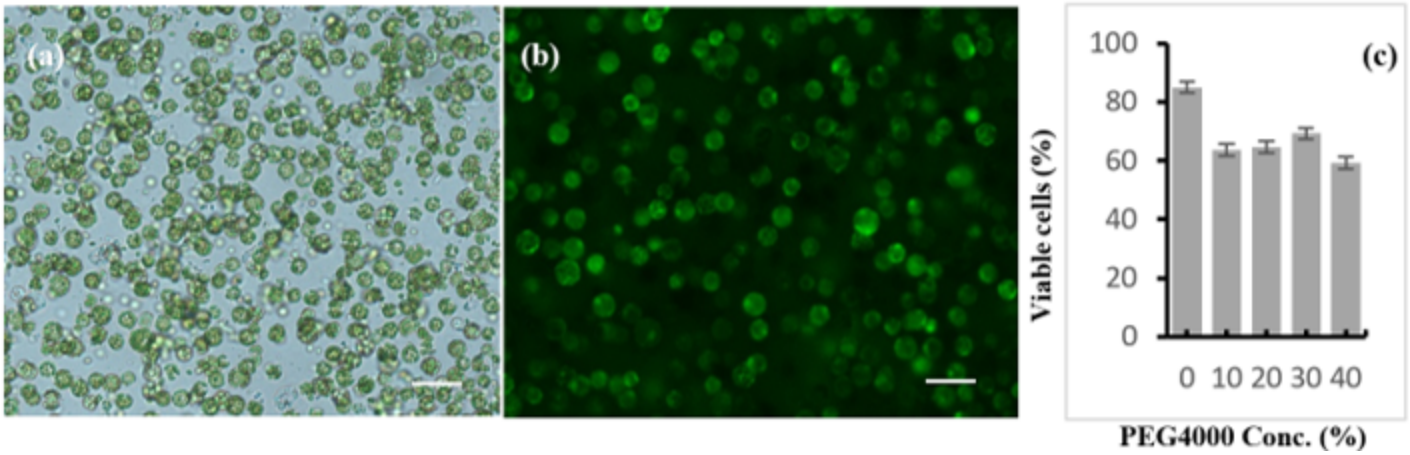

Figure 1

Viability experiment with PEG4000. (a & b) The viability of millet protoplasts was observed with FDA fluorescence treated with 30% (w/v) PEG4000 **(c)** The viability of protoplasts with different concentrations of PEG4000 solutions (scale bars indicate 50 μm).

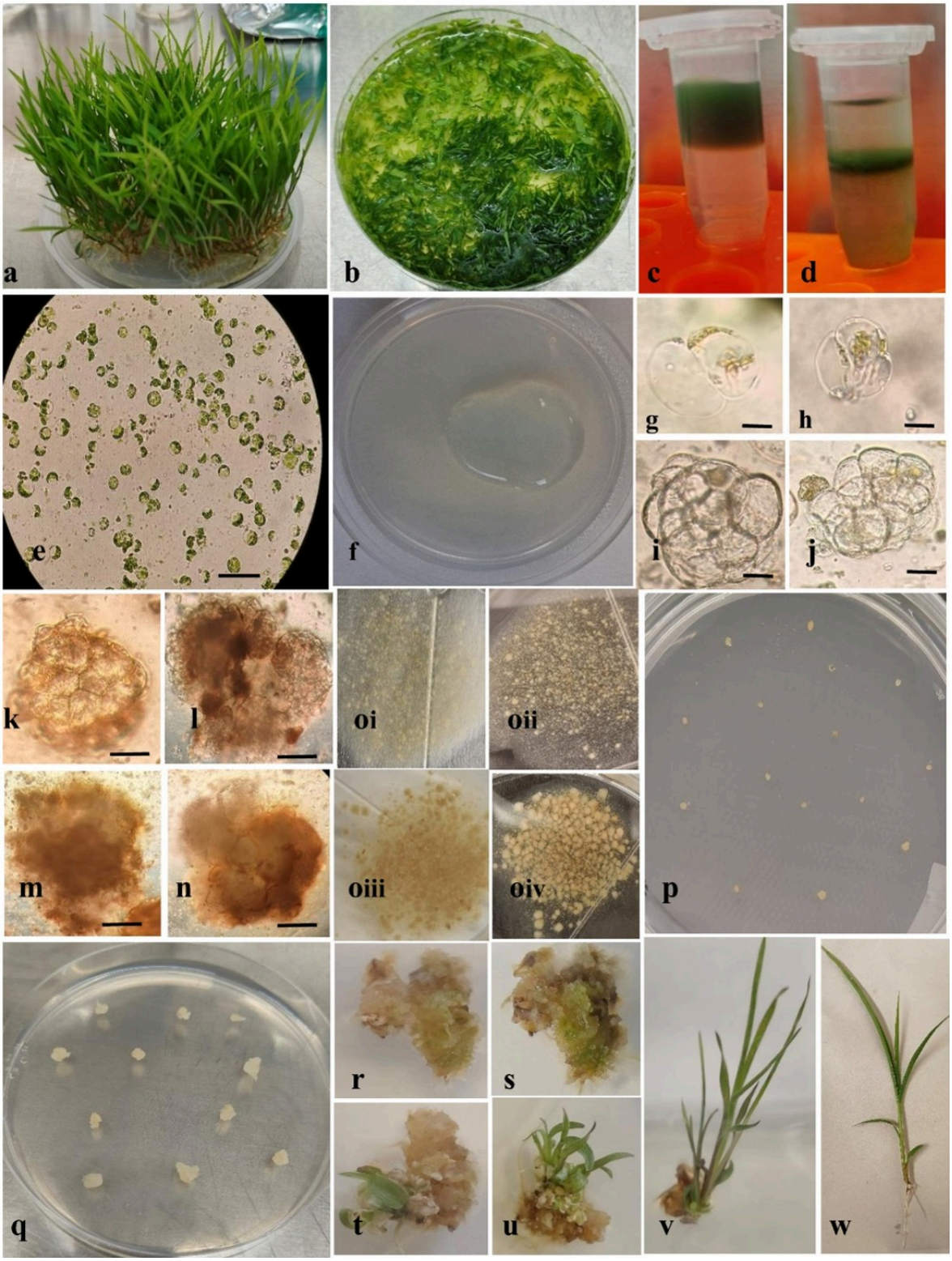

Figure 2

Plantlet regeneration from foxtail millet protoplasts. **a**: Fourteen day-old seedlings of stem and sheath leaves for protoplast isolation; **b**: Incubation of tissue for plasmolysis; **c-e**: the interface purification method of protoplasts on 0.5M sucrose gradient solution; **f**: Embedding of protoplasts in sodium alginate thin layer; **g**: First cell division (two cell structure); **h**: Second cell division (four cell structure); **i**: Eight-cell structure; **j**: Multi-cell aggregate; **k-n**: Incubation of callus cultures for 3-4 weeks on CCM; **o(i-**

iv): Alginate layers with overgrown callus colonies differing in size; **p**&**q**: Proliferation of callus for 2-3 weeks on CCM+PSK; **r**-**u**: Organogenesis from protoplast-derived cells for 3-4 weeks in SRM+PSK; **v**&**w**: Protoplast-derived plant ready for *ex vitro* acclimatization. Scale bars: 50 µm (e, g–j), 0.5 cm (k–n).

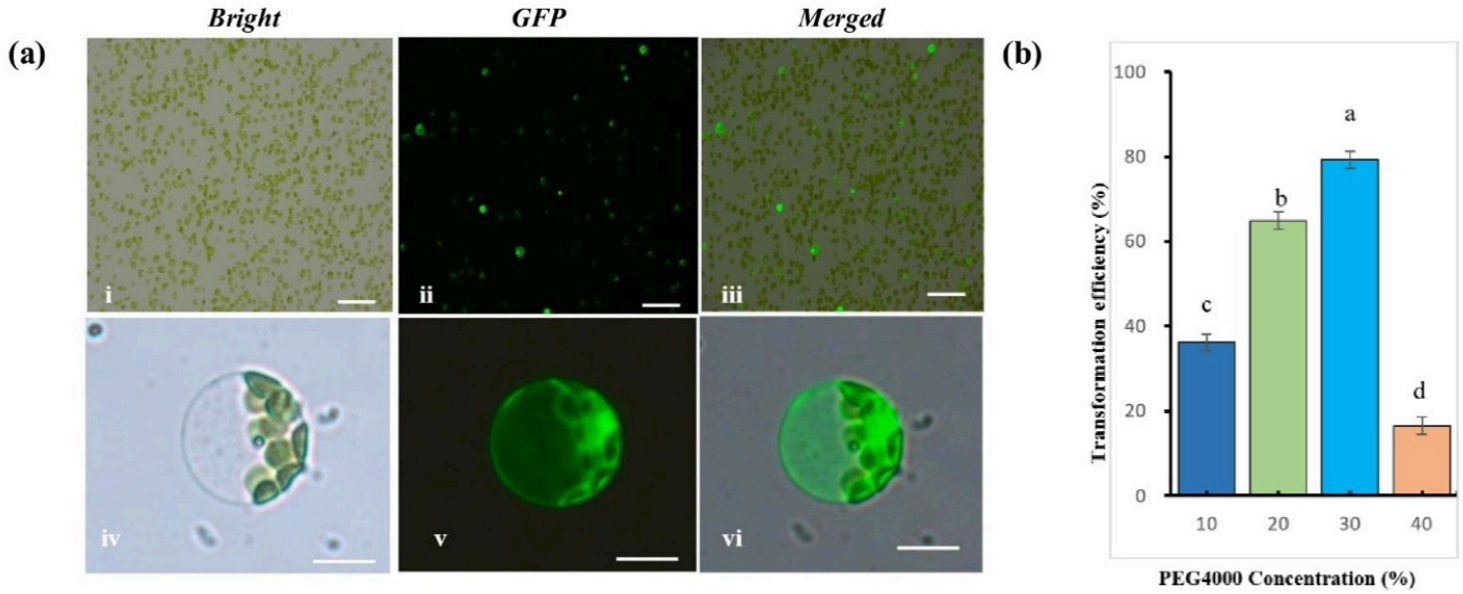

Figure 3

***GFP* transient expression in *Setaria italica* L. protoplasts. (a)**Transfected protoplasts expressing GFP signal with PEG 30% (w/v) after 30 minutes of incubation: i–iii (The scale bars = 100 µm); iv–vi (The scale bars = 20 µm). **(b)**Protoplast transfection efficiency with different concentrations of PEG4000 solutions. The different letters indicate significant differences (P ≤ 0.05) according to the HSD test.

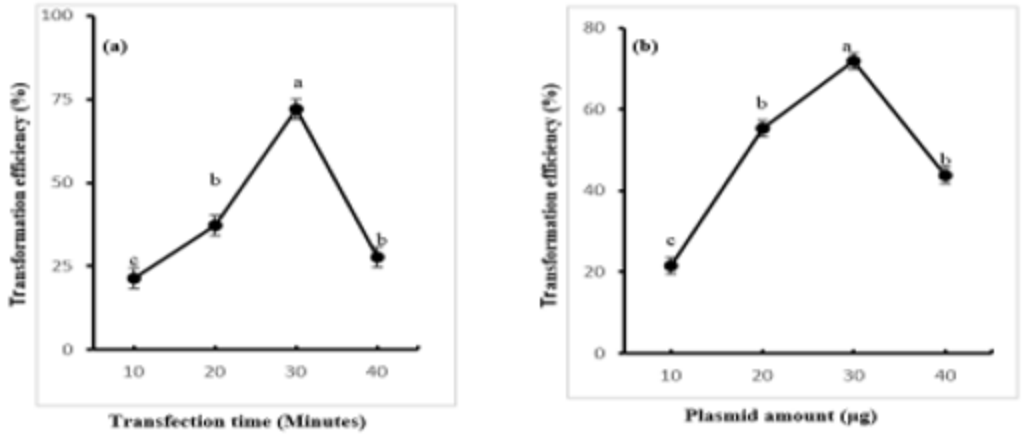

Figure 4

Efficient transfection of foxtail millet protoplasts. Effects of **(a)** transfection time and **(b)** plasmid amount on protoplast transformation efficiency. Values are expressed as mean ± SE, the error bars

represent at least three independent replicates. The different letters indicate significant differences ($P \leq 0.05$) according to the HSD test.

Supplementary Files

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- Table15.docx