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In vitro plant regeneration and callus formation in Proso millet (*Panicum miliaceum* L.)

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Abstract
Proso millet (*Panicum miliaceum* L.) is a significant crop that is extensively cultivated in India due to its high nutritional value and a multitude of health benefits. This hardy grain is particularly esteemed for its capacity to thrive in adverse environmental conditions, including elevated temperatures, drought and suboptimal soil quality. A study was carried out to investigate *In vitro* plant regeneration, focusing on root and shoot development, as well as callus induction, from the seeds of *Panicum miliaceum* L. (var GPUP-25) where the seeds were cultured on MS media supplemented with various concentrations of NAA and 2,4-D at concentrations of 1.5 mg/l, 2.0 mg/l, and 2.5 mg/l. Additionally, subculturing was conducted utilizing explants (leaf bases) from four-week-old seed-derived plantlets, initially grown on basal MS media as well as MS media supplemented with 1.5 mg/l NAA, and then transferred to MS media containing varying concentrations of NAA, 2,4-D, IAA, and IBA to facilitate direct plant regeneration. The most favorable root development occurred in MS media supplemented with 1.5 mg/l NAA, which resulted in the highest number of roots relative to the other concentrations of NAA as well as 2,4-D. In the case of shoot development, minor variations were observed in both the quantity and length of shoots across all concentrations of NAA and 2,4-D. The optimum callus formation was induced with MS media supplemented with 2.5 mg/l 2,4-D. Callus formation was also supported through the subculturing of *In vitro* grown proso millet on MS media supplemented with 2.5 mg/l NAA.

Keywords: Proso millet, *Panicum miliaceum* L., NAA, 2,4-D, plant regeneration, callus induction

Introduction
Millets are a group of annual, small-seeded cereal crops that belong to the Poaceae family. They are cultivated globally for various purposes, including food, livestock feed, forage, and fuel production [1]. They are regarded as one of the earliest cereal crops cultivated since the dawn of human civilization [2, 3]. Originally cultivated in Asia and Africa, millets later became crucial food sources for developing civilizations worldwide [4]. These crops are classified into two categories: major millets and minor millets. Major millets encompass widely recognized varieties such as pearl millet, finger millet, and sorghum [5, 6], which dominate production in many regions due to their adaptability and yield potential. In contrast, minor millets, often referred to as small millets, comprise a diverse assortment of ancient grains, including varieties such as foxtail millet, barnyard millet, proso millet, browntop millet, kodo millet and little millet [5, 6] which have been cultivated for centuries, primarily in marginalized and arid regions, and are now gaining attention due to their exceptional nutritional profiles, which often exceed those of conventional cereals. In light of the contemporary global phenomenon of climate change which includes rising temperatures, altered precipitation patterns, and an increased frequency and severity of extreme weather events, along with rising sea levels it has become evident that the yields of major staple cereals such as rice, wheat, and maize are declining. This decline necessitates the integration of millets into agricultural production systems. Millets, being C4 plants, exhibit superior photosynthetic efficiency, shorter growth duration, higher dry matter production capacity, and a notable tolerance to both heat and drought, positioning them as a viable alternative in changing climate scenarios [7, 8, 9]. Renowned for their high protein content, these millets serve as a crucial food source for humans, animals, and birds. In addition to their protein richness, they boast essential nutrients such as vitamins, minerals, and dietary fibers [10, 11, 12]. Moreover, they possess remarkable biological properties, including anti-inflammatory, antimicrobial, antiulcer, and anti-cancerous effects, as well as the ability to lower cholesterol and glucose levels in the body [13, 14, 15, 16]. Additionally, small millets are extensively utilized for the production of starch and alcohol, thereby contributing to the heightened demand for these versatile grains [11].

Despite these impressive attributes, the global consumption and production of small millets are declining, potentially due to the widespread availability and convenient cooking methods of other staple cereals like rice, wheat, and maize [11, 12].

Proso millet (*Panicum miliaceum* L.), commonly recognized by names such as broomcorn millet (China), common millet (USA, Japan and Korea) and hog millet in the USA [17, 18] is a significant and versatile minor millet cultivated extensively across India. In India, proso millet is known by a variety of names in local languages as Cheena and Bari in Hindi, Panivaragu in Tamil and Malayalam, Variga in Telugu, Baragu in Kannada, Vari in Marathi, Cheena in Bengali and Marathi, Cheno in Gujarati, while in Oriya, it is named China [12]. These diverse names reflect the millet's deep cultural integration and varying importance across different regions. Proso millet is believed to have originated in the Manchurian region of China approximately 10,000 years ago [9, 18]. Today, it is widely grown in various regions across the globe, including northwest China, southern and central parts of India, Australia, the United States, and several countries in Europe. As the third most important millet crop, following pearl millet and foxtail millet, proso millet plays a significant role in global food security and agricultural diversity [9]. In India, proso millet is primarily cultivated in Madhya Pradesh, Maharashtra, Eastern Uttar Pradesh, Bihar, Tamil Nadu, Andhra Pradesh and Karnataka [19]. This resilient grain is particularly valued for its ability to thrive in challenging environments, including high temperatures and poor soil conditions, making it an ideal choice for regions where other grains may struggle. As a warm-season crop, proso millet has a notably short growing cycle, typically maturing in about 60 to 90 days, which allows for multiple harvests in a single growing season, enhancing productivity and food supply. Additionally, its low moisture requirements make it an excellent option for cultivation in arid and semi-arid regions, where water resources might be limited [1, 20]. Proso millet has high nutritional value and offers a wide range of health benefits. It serves as a rich source of minerals, dietary fiber, polyphenols, vitamins, and proteins, making it an indispensable addition to the diet. Its gluten-free status makes it particularly advantageous for individuals with gluten intolerance [1]. This grain is not only abundant in essential vitamins such as niacin, B-complex vitamins, and folic acid, but also in vital minerals including phosphorus, calcium, potassium, sodium, magnesium, manganese, zinc, and iron. Thus, this grain is beneficial for preventing Pellagra, a condition caused by a deficiency of niacin, or Vitamin B3 [4, 21]. Furthermore, these minerals are essential for the maintenance of healthy blood vessels, bones, nerves, and immune function. Additionally, they help alleviate respiratory issues and heart-related conditions, while also reducing the frequency of migraine attacks [9]. Proso millet delivers essential amino acids, particularly methionine and cysteine, further enhancing its nutritional profile. Furthermore, proso millet boasts high lecithin levels, which are crucial for optimal neurological health [1]. With a low glycemic index, proso millet significantly reduces the risk of type-2 diabetes and cardiovascular disease [22]. Studies have highlighted the presence of phytochemicals including such as phenolics and flavonoids owing to the antioxidant [23] and the potential antiproliferative properties of proso millet, explaining their role in inhibiting the growth and reproduction of cells, a characteristic of significant interest in cancer research [24-26]. Furthermore, proso millet has been used traditionally as a recuperative food, especially beneficial for women following pregnancy and individuals recovering from illness, due to its wholesome nutritional profile [27].

Renowned for its rich nutritional profile, encompassing carbohydrates, protein, essential nutrients, and gluten-free properties, proso millet, despite its notable attributes, often experiences neglect due to limited genetic diversity and suboptimal productivity [28]. Despite the nutritional superiority, drought tolerance, and adaptability to marginal conditions, the cultivation of small millets is experiencing a continual decline. Enhancing productivity is crucial to bolster competitive capabilities in marginal agro-ecologies and to stimulate demand, particularly among urban elites, to prevent further regression. Improving productivity and tailoring cultivars to specific location-based requirements are essential. Enhancing nutrient-use efficiency, developing dwarf varieties, and improving water-use efficiency are key objectives to address these challenges [12]. These objectives can be achieved by harnessing the technology of genetic engineering [29]. Thus, an efficient *In vitro* regeneration system is essential for the genetic transformation and recovery of transgenic millet plants [30, 31]. Transgenic crop plants with desired traits can be produced through tissue culture techniques [32]. In recent years, tissue culture techniques have been increasingly employed to enhance the propagation and genetic improvement of millets. Noteworthy research suggests the promise of enhancing proso millet through advanced tissue culture methodologies, presenting prospects for the development of improved cultivars. In a pioneering study, Rangan (1974) first reported the successful induction of callus tissue from the mesocotyls of proso millet [33]. Subsequent research expanded on Rangan's work, with Heyser and Nabors (1982) inducing callus from stem segments [34], and Rangan and Vasil (1983) focusing on immature inflorescences [35]. *In-vitro* regeneration of proso millet has been accomplished using mature embryos, immature embryos, and immature inflorescence on Murashige and Skoog medium [36]. Additionally, Bobkov (2007) achieved callus induction from anthers of proso millet [37].

The growth and morphogenesis of plant tissues under *In vitro* conditions are highly influenced by the specific composition of the culture medium. The culture medium, which is a concoction of various nutrients, vitamins, and growth regulators, plays a critical role in determining the success of plant tissue culture. The selection of an appropriate medium formulation is essential for establishing an efficient tissue culture system for plant regeneration. The growth rate of plant tissues cultured *In vitro* is intricately linked to the net assimilation rate, which is the result of the uptake of essential nutrients, minerals, and other growth-promoting substances from the medium. Therefore, understanding the role of the culture medium and its constituents is fundamental in optimizing plant tissue culture systems for successful regeneration and growth [38]. Hence, the overarching aim of the present study is to evaluate the potential of *Panicum miliaceum* L. for its *In vitro* regeneration by supplementing different auxins on nutrient media and the seeds for its *In vitro* germination. Moreover, a comparison was also drawn between the *In vivo* and *In vitro* grown seedlings for the root and shoot lengths. Seeds and the resultant *In vitro* grown seedlings were used as explants for the morphogenic studies.

Materials and Methods

Culture media

In the present investigation, basal media was used which consisted of MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. All the chemicals used for the media preparation were of analytical grade (Hi-media). The desired media volume was achieved by adding distilled water followed by homogenization and continuous stirring. The pH was adjusted to 5.8 using 0.1N NaOH and 0.1N HCl. The basal media was supplemented with different phytohormones i.e. Naphthalene acetic acid (NAA) and 2,4 dichlorophenoxyacetic acid (2,4-D) in different concentrations (1.5, 2.0 and 2.5 mg/l) separately for root and shoot regeneration and callus formation. Culture tubes containing the media were autoclaved under pressure of 1.1kg/cm^3 at 121°C for 15-20 minutes.

Plant material

Seeds of *Panicum miliaceum* L. (var GPUP-25) were procured from Birsa Agricultural University, Ranchi, Jharkhand. The seeds were washed with tap water followed by rinsing with distilled water to remove any impurities and debris. The seeds were then immersed in diluted teepol for 5-6 minutes followed by rinsing with distilled water 2-3 times. For surface sterilization, the seeds were treated with 70% ethanol for 1-2 minutes and further immersed in 0.1% mercuric chloride ($HgCl_2$) for 3 minutes followed by 3 rinses in autoclaved distilled water under aseptic conditions in the laminar airflow cabinet. The seeds were then kept in autoclaved Whatman filter paper to dry off the excess moisture and were made ready for inoculation.

Culture conditions

All the experiments were carried out under aseptic conditions. Before inoculation, the culture area, inoculation tools and glassware were sterilized with UV light and the surface of the laminar air flow hood was swabbed with 70% ethanol. The seeds, used as explants were inoculated onto the culture media and incubated in the culture room maintained in diffuse fluorescent light at temperature 25±2 °C and 50-60% humidity. Each experiment was done in triplicates.

Pot conditions

The seeds were soaked in tap water for approximately 8 hours before sowing it in pots. A mixture of sand and soil without any fertilizers was used for sowing. The pots were kept under mild sunlight conditions and were sprinkled with water once a day and were left to germinate.

Subculture

Explants (leaf base) from 4 weeks old seed derived, basal MS media as well as MS+1.5 mg/l NAA grown plantlet, were excised with the help of a scalpel under aseptic condition inside a laminar airflow hood and were sub-cultured on MS media supplemented with different concentrations of NAA, 2,4-D, IAA, IBA alone and in combination for direct plant regeneration.

Data Analysis

Data were collected at the interval of 7 days for over 4 weeks. The data was analyzed statistically by mean and standard deviation.

Results and Discussion

Rooting Response

The present study revealed that the most optimal conditions for root development were present in the MS media supplemented with 1.5 mg/l of NAA (42.33±2.516), which yielded the highest root count on 28th day in comparison to the other two tested concentrations of NAA (2 mg/l and 2.5 mg/l). Additionally, roots observed in 1.5 mg/l NAA concentration, not only exhibited a higher quantity but also demonstrated considerable length, signifying robust and vigorous root growth. However, an intriguing inverse relationship was noted, as the concentration of NAA increased, the number and length of roots declined (Table 1). Conversely, it was observed that the thickness or diameter of the roots increased with higher NAA concentrations, suggesting that NAA stimulated initial root formation which may have restricted further elongation (Fig. 2). In contrast, the effects of 2,4-D on root development were markedly less favorable (Fig. 2). In MS media supplemented with various concentrations of 2,4-D, root formation was found to be minimal. Throughout the initial two weeks, there were no observable roots at any of the tested concentrations. As the experiment progressed to the 21st day, a limited number of roots appeared, yet they were significantly shorter (2.46±0.542) than those formed in 1.5 mg/l NAA (23.66±1.154) concentration (Table 1). This finding highlights the differential influences of the two growth regulators and their unique biological mechanisms. Interestingly, while NAA inhibited root proliferation at higher concentrations, the study revealed that with increasing concentrations of 2,4-D, there was a positive correlation with the number and length of roots. This phenomenon indicates the potential of 2,4-D to stimulate root induction under certain conditions. When compared to the control which utilized basal MS media devoid of any growth regulators, the addition of NAA clearly facilitated superior root development over MS media as well as 2,4-D, indicating the crucial role of these regulators in enhancing root development (Fig. 2). Moreover, a pronounced variation was noted in the morphology of the roots depending on the medium used. Specifically, roots grown in basal MS media exhibited a well-defined tap root system, characterized by a dominant central root structure that extended deep into the media along with distinct lateral branches (Fig. 1). In contrast, roots cultivated in MS media supplemented with NAA displayed a prolific formation of fibrous roots, indicating a different developmental response likely influenced by the presence of this auxin (Fig. 2). This divergence in root morphology highlights the impact of growth conditions and different hormonal concentrations on the overall development of proso plant roots which leads to further investigation into the underlying mechanisms. These observations contribute essential knowledge to the field of plant tissue culture and highlight the importance of optimizing *In vitro* conditions to enhance overall plant development.

A comparison was also drawn between the root development under *In vivo* and *In vitro* conditions. Plantlets were observed under *In vivo* conditions within 3-4 days of sowing. Mature seedlings were observed within 11-12 days after germination. The *In vitro* germination of *Panicum miliaceum* L. was seen after 5-7 days of seed inoculation. The seeds germinated rapidly under *In vitro* conditions and formed plantlets in control media i.e. MS media without any growth hormones. After 7 days of inoculation, the seeds gave out shoots and roots with no lateral branches. Upon observation in the later

days that is in the 3rd and 4th week, the tap root system developed along with shoot elongation. There was a clear distinction between the roots of proso plantlets grown *In vivo* and those grown *In vitro* (on basal MS media). Notably, the roots developed under *In vitro* conditions displayed a markedly higher number of lateral roots than their *In vivo* counterparts (Fig. 1&2). This finding is significant as it suggests the influence of various environmental or hormonal factors that may be at play in the *In vitro* conditions, leading to enhanced lateral root development.

Shooting Response

The analysis also included examining shoot development, where the findings indicated minimal variation in the number of shoots with different concentrations of NAA and 2,4-D. This suggests that under the specific experimental conditions, both growth regulators had a limited impact on shoot growth. However, regarding the lengths of shoots, it was evident from the results that with increasing concentration of NAA, the lengths of shoots decreased while it was vice versa in the case of 2,4-D (Fig. 1&2).

Table 1: Effect of different concentrations of auxins supplemented to MS media on root and shoot development of *Panicum miliaceum* L. (var GPUP-25) seed culture

Media concentration and combination	No. of days	Root development		Shoot development	
		No. of roots Mean± S.D.	Length of roots (in cm) Mean± S.D.	No. of shoots Mean± S.D.	Length of shoots (in cm) Mean± S.D.
MS basal	7	1.33±0.577	1.33±0.057	1.3±0.577	1.46±0.152
	14	3.66±1.154	2.03±0.057	1.3±0.577	4.13±0.152
	21	7.33±0.577	4.13±1.152	2.33±0.577	5.16±0.152
	28	14.33±1.154	4.76±0.115	2.33±0.577	5.5±1
MS+1.5 mg/l NAA	7	4.33±0.577	2.26±0.251	2.66±0.577	4.23±0.251
	14	14.66±0.577	3.06±0.115	3.33±0.577	6.46±0.208
	21	23.66±1.154	3.53±0.152	4.33±0.577	8.33±0.351
	28	42.33±2.516	4.1±0.173	4.66±0.577	9.96±0.251
MS+2.0 mg/l NAA	7	3.32±0.672	1.96±0.147	2.67±0.562	4.01±0.238
	14	13.82±0.675	2.75±0.154	3.32±0.562	6.12±0.245
	21	22.88±1.472	3.33±0.115	4.34±0.561	7.59±0.241
	28	38.76±2.516	3.50±0.121	4.64±0.558	9.12±0. 232
MS+2.5 mg/l NAA	7	2.73±0.577	1.21±0.115	2.66±0.577	3.82±0.214
	14	10.24±0.577	1.73±0.251	3.34±0.564	5.98±0.154
	21	18.32±1.154	1.89±0.152	4.33±0.577	7.21±0.154
	28	30.48±0.577	2.05±0.121	4.65±0.562	8.87±0.154
MS+1.5 mg/l 2,4-D	7	-	-	0.63±0.558	0.82±0.251
	14	-	-	1.12±0.577	1.76±0.243
	21	2.46±0.542	0.12±0.062	1.13±0.564	2.73±0.245
	28	2.98±0.672	0.23±0.062	1.16±0.556	3.21±0.241
MS+2.0 mg/l 2,4-D	7	-	-	0.64±0.561	0.98±0.238
	14	-	-	1.22±0.562	1.82±0.245
	21	4.22±0.761	0.48±0.143	1.23±0.577	2.86±0.246
	28	4.67±0.734	0.97±0.157	1.23±0.542	3.42±0.245
MS+2.5 mg/l 2,4-D	7	-	-	0.66±0.577	1.266±0.208
	14	-	-	1.33±0.577	2.03±0.251
	21	7.10±0.513	0.62±0.154	1.3±0.577	3±0.2
	28	7.12±0.526	1.23±0.157	1.3±0.577	3.53±0.152

Values are Mean± S.D of three independent experiments each with three replicas.
S.D. = Standard deviation

Callus induction and Subculture

The basal MS media without any hormone did not support callus growth. Notably, callus formation was observed in the MS media containing 2.5 mg/l of 2,4-D, where abundant callus tissue was observed from the seeds (Fig. 2). Several other studies have also utilized seeds for the induction of callus.[38&39] The findings of the present investigation corroborate with the study done by Liu *et al*., 2021 where they reported no callus induction on MS medium without 2,4-D. The callus induction observed in their study was markedly higher at a concentration of 3.0 mg/l of 2,4-D compared to the other concentrations examined (0.0, 1.0, 2.0, and 4.0 mg/l). Furthermore, their results demonstrated that both excessively high and excessively low concentrations of 2,4-D resulted in a decreased rate of callus induction, suggesting that there is a critical range for effective callus formation. At higher concentrations, particularly beyond the optimal 3.0 mg/l, there was also a significant observation of callus browning, which may indicate stress responses in the tissue or potential cytotoxic effects resulting from the elevated levels of the growth regulator. This browning may imply the viability of the induced callus and lead to further investigation into the effects of 2,4-D concentration on tissue culture responses.[31] In 1983, Rangan and Vasil successfully established embryogenic callus cultures derived from the young inflorescence segments of proso millet and little millet. The initiation of these cultures was done using MS medium supplemented with 5% coconut milk and 2,4-D at a concentration of 2.5 mg/l [35]. Jain *et al*., 2001 cultured mature embryos, immature embryos, and immature inflorescences were cultured on MS medium supplemented with 2,4-D, either alone or in combination with Kinetin (Kn) or Benzylaminopurine (BAP). The immature embryos produced moderate amounts of callus when grown on MS medium containing 2,4-D (0.5-5 mg/l) and Kn (0.2-1 mg/l). Conversely, when 2,4-D (0.5-5 mg/l) was used alone on the MS medium, only minimal was formed.[36] However, in the present study, callus was successfully formed in 2,4-D alone. Pande *et al*., 2015 studied the formation of organogenic calli by culturing finger millet seeds on MS media supplemented with varying concentrations of 2,4-D, (0.5, 1.0,

1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mg/l). Their findings demonstrated that the MS media enriched with 1.5 mg/l of 2,4-D resulted in the highest frequency of callus induction.[30] Furthermore, the present study demonstrated callus development along with plant regeneration occurred when the explants from proso millet, initially grown on 1.5 mg/l NAA-supplemented MS media, were subsequently subcultured onto MS+2.5 mg/l NAA (Fig. 2). This observation points to the sustained potential for callus activity and growth, following initial NAA exposure. Liu *et al*., 2021 also found NAA to be suitable for the initiation of callus from young leaves.[31] Haridas and Ashok, 2017 standardized an efficient protocol for callus induction, subsequent growth, and *in-vitro* regeneration in six genotypes of proso millet revealing that callus induction was successful with both 2,4-D and NAA. Mature embryos (seeds) of proso millet were cultured on MS medium supplemented with varying concentrations of 2,4-D (0.1-2.0 mg/l) and NAA (1.0-4.0 mg/l) to facilitate callus induction and subsequent growth. The highest rate of callus induction occurred at a concentration of 2 mg/l of 2,4-D, followed by 1 mg/l and 0.5 mg/l. No callus formation was observed with the addition of 0.1 mg/l of 2,4-D. With NAA, the maximum callus induction response was recorded at 3 mg/l, followed by 4 mg/l and 2 mg/l while the lowest response was observed at 1 mg/l of NAA [28].

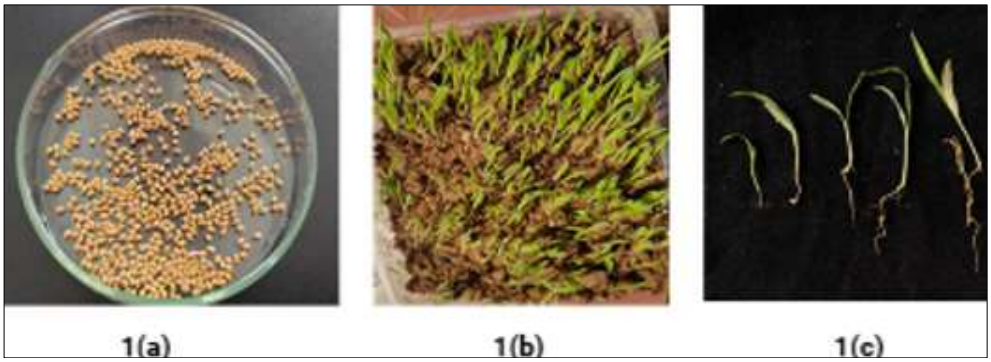

Fig 1: (a) Seeds of *Panicum miliaceum* L. (var GPUP-25) (b) *In vivo* grown plantlet of proso millet in pot condition (c) Root and shoot development of *In vivo* grown proso plantlets over a period of 28 days

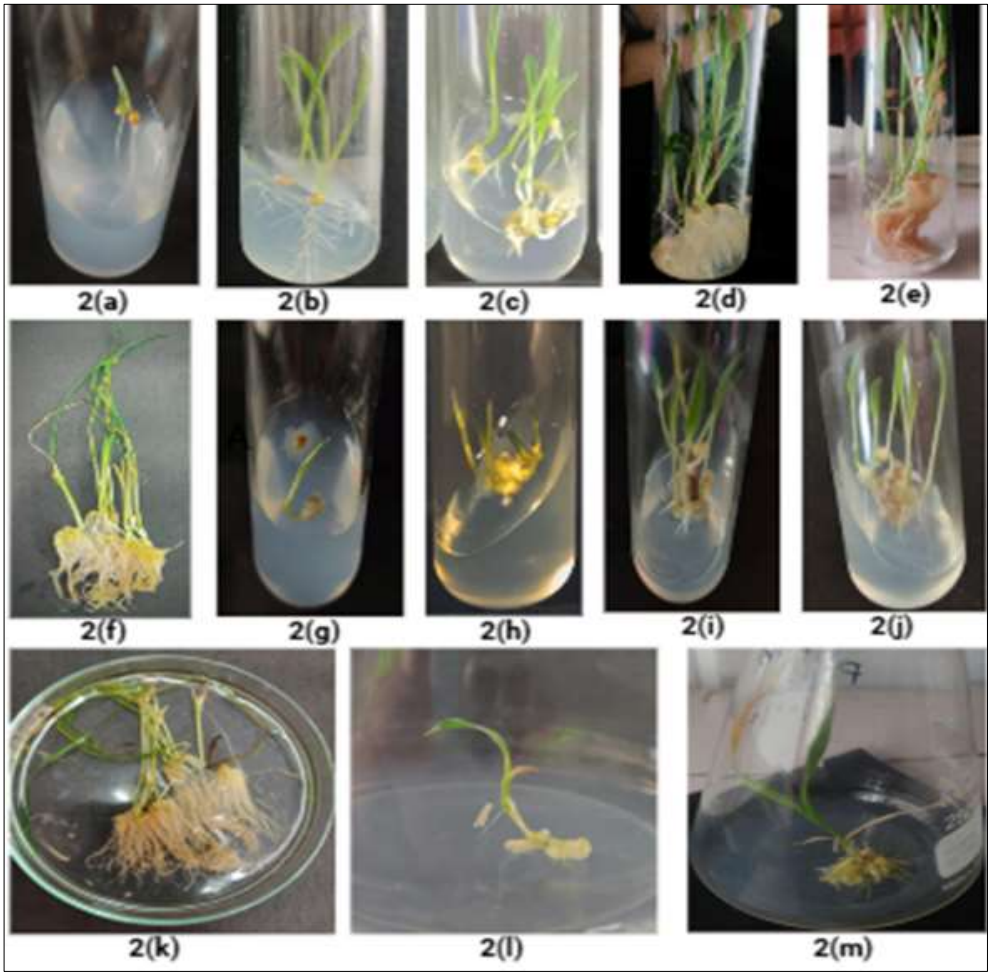

Fig 2: (a) (b) Root and shoot induction on basal MS media on 7th day and 28th day respectively (c) Root and shoot induction on MS+ 1.5 mg /L NAA on 7th day (d) (e) (f) Proso seed culture on MS+1.5 mg/l NAA showing profuse fibrous root along with shoot formation on 14th, 21st and 28th day respectively (g) (h) (i) (j) Simultaneous plant regeneration and callus induction in MS+2.5mg/L 2,4-D on 7th, 14th, 21st and 28th day respectively (k) 28 days old proso plantlet seed cultured on MS+1.5 mg /l NAA used as explant for subculture (l) (m) Simultaneous plant regeneration and callus induction upon subculturing on MS+2.5 mg/l NAA

Conclusion

This study employed a detailed temporal observation methodology, providing insights into the developmental dynamics of roots and shoots over time. Experiments were conducted over a four-week period to investigate the *In vitro* development of roots and shoots, as well as the formation of callus. In the present study, root development was best reported in MS media supplemented with 1.5mg/l NAA with the highest number of roots in comparison to the other two concentrations of NAA. In comparison with the control (basal MS media) and MS supplemented with 2,4-D, better root development was seen in NAA. The study also highlighted the difference in root morphology of *In vivo* and *In vitro* grown proso millets, later having a greater number of lateral roots. Moreover, the roots typically found within the Poaceae family are known to be either fibrous or adventitious. However, this study revealed a notable exception, the roots observed in the basal MS media exhibited a tap root structure accompanied by lateral roots. This finding is significant as it highlights a divergence from the common root characteristics associated with this plant family, potentially offering new insights into their growth patterns and development in controlled environments. Unlike roots, slight variation in the number as well as the length of shoots was observed in both NAA and 2,4-D at different concentrations. Callus formation was best observed in MS media supplemented 2.5 mg/l 2,4-D among all the different concentrations. Callus formation was also supported upon subculturing *In vitro* grown proso millet on MS media supplemented with 2.5 mg/l NAA. The present investigation revealed that the number and length of roots and shoots consistently increased as the experimental duration progressed, illustrating the dynamic nature of plant development in response to applied growth regulators. The findings indicated that varying concentrations and combinations of plant growth regulators supplemented with MS media produced distinct effects on root number, root length, shoot number, shoot length, and callus induction.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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