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Research article

# *In-vitro* regeneration of *Sehima nervosum*: an important range grass species

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## Abstract

*Sehima nervosum* (Rottler) Stapf is an apomictic, polyploid, and perennial natural range grass found inherently rich in precursors for several industrially important biomolecules. Production of nutraceuticals (prebiotics xylo-oligosaccharides) from this grass is promising. However, improvement efforts suffer due to its narrow genetic base and limited variability in germplasm collections. Biotechnological interventions promise to enhance genetic variability by inducing somaclonal variations and developing sexually reproducing lines to facilitate the release of otherwise 'frozen' variability. Seeds of *S. nervosum* cv. *Bundel Saen Ghas 1* was used as an explant to develop a high-efficiency reproducible regeneration protocol in this crop. The seeds were inoculated onto MS media supplemented with different concentrations of 2,4-D for callus induction. Various combinations of BAP and Kinetin were tested for shoot regeneration from calli, while combinations of NAA were tested for efficient rooting. Callus induction frequency was maximum (upto 95%) in a medium containing 3.5 mg/l 2, 4-D. The highest shoot induction (98%) was obtained when supplemented with 2.5 mg/l kinetin, while the highest root initiation (42%) was obtained with 4.5 mg/l NAA supplementation. Regenerated plantlets were transplanted to pots, where they exhibited morphologically normal growth. A high-efficiency reproducible protocol for *in-vitro* culture was developed in this grass. This in-vitro tissue culture protocol could help generate somaclones and genetic transformation of *Sehima* with genes of agronomic importance.

**Keywords:** Apomictic grass, Callus, Perennial range grass, Regeneration frequency, Tissue culture

## Introduction

*Sehima nervosum* is one of the important rangeland grasses in India. It is commonly referred to as *Saen* grass in India and white grass in Australia, and it has also been reported from the central geographical area of Sudan (Samanta *et al.*, 2012). This grass has high nutritive value and is employed for grazing and hay preparation for quality fodder (Purohit and Kukda, 1995). It prefers hot and dry climates and survives even in limited rainfalls. As this natural grass is found inherently rich in precursors for several industrially important biomolecules, fractioning those precursors seems promising. Production of nutraceuticals (prebiotics xylooligosaccharides) from the lignocellulosic biomass of this grass is promising, as this grass does not compete with food crops and is relatively cost-effective than conventional agricultural feedstocks (Samanta *et al.*, 2012). However, the germplasm of this grass has narrow genetic variability (Roy *et al.*, 2022). Genetic improvement (Roy *et al.*, 2019) and nutrient digestion, fermentation, gas production and partition factor of Sehima in ruminant diets is well documented (Singh *et al.*, 2015). Being largely apomictic in reproduction, the generation of variability through hybridization has been extremely limited. The utilization of biotechnological tools is one of the potential ways to introduce variability and transfer desirable traits.

Detailed studies on tissue culture have been reported in most commonly used valuable range grasses in pasture, such as *Cenchrus* (Sankhla and Sankhla, 1989; Kackar and Shekhawat, 1991; Batra and Kumar, 2002; 2003; Yadav *et al.*, 2009; Kumar and Bhat, 2012; Dwivedi *et al.*, 2016) and *Dichanthium* (Gupta *et al.*, 1997; Gupta *et al.*, 1998; Bhat *et al.*, 2001; Dalton *et al.*, 2003; Kumar *et al.*, 2005). In contrast, little work has been done on *Sehima* (Purohit and Kukda, 1995) and *Heteropogon* (Purohit and Kukda, 1996). Here a detailed study on *in-vitro* callus induction and regeneration is reported, which relies on embryogenesis

and organogenesis from mature embryo explant. Indeed, the present investigation deals with various factors affecting callus induction and the subsequent formation of shoots and roots.

## Materials and Methods

In this study, different medium supplements were optimized, and the effects of various concentrations and combinations of phytohormones on callus induction and plantlet regeneration were recorded. The study was conducted for two consecutive years (2021-23) at ICAR-Indian Grassland and Fodder Research Institute, Jhansi, India.

***Callus induction and embryogenesis*:** Mature and healthy seeds of *S. nervosum* (Var. Bundel Saen 1) were used as explant materials. These mature seeds were manually dehusked, surface sterilized with 70% (*v/v*) ethanol for 1-minute, rinsed in sterile distilled water, and dipped in 0.1% (*w/v*) mercuric chloride solution for 10 minutes, followed by five or six rinses with sterile distilled water. The surface sterilized seeds were inoculated onto Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) containing different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mg/l) of 2,4-D (2,4-dichlorophenoxyacetic acid) (Himedia, Mumbai, India) for callus induction. A total 09 media combinations were used. The pH of MS basal medium, which contained 30 $gL^{-1}$ sucrose (Himedia), was adjusted to 5.9 before being solidified with 0.7% (*w/v*) agar (Himedia) and autoclaved at 121°C for 15 minutes. Culture plates (90 mm Petri plates) were cultured at 26 ± 2°C in the dark for four weeks for callus induction. Observations were recorded on callus induction frequency (divide the number of seeds with callus by the number of seeds inoculated and multiply by 100) after 18 days, and calli were sub-cultured in the same medium for embryogenesis.

***Shoot regeneration*:** The embryogenic callus was cultured onto a shoot regeneration media which contained Murashige and Skoog (MS) basal medium with different concentrations (0.5–3.0 mg/l) of Kinetin (Himedia, Mumbai, India), BAP (0.5–3.0 mg/l), and combination of Kinetin+BAP (0.5–2.5 mg/l) at 25◎ under an alternative photoperiod scheme (16 hours light/8h dark) for shoot regeneration. A total of 17 media combinations (MS+0.5Kin, MS+1.0Kin, MS+1.5Kin, MS+2.0Kin, MS+2.5Kin, MS+3.0Kin, MS+0.5BAP, MS+1.0BAP, MS+1.5BAP, MS+2.0BAP, MS+2.5BAP, MS+3.0BAP, 0.5Kin+0.5BAP, 0.5Kin+1.5BAP, 0.5Kin+2.5BAP, 0.5BAP+1.5Kin, 0.5BAP+2.5Kin) were tested. For each treatment, 15 to 20 embryogenic calli were planted on regeneration media in three conical flasks (100 mL) as one replicate, with three replicates performed. Greenish callus and shoot elongation started after three weeks on the same media and culture conditions. Shoot regeneration frequencies were recorded after 21 days of embryogenic callus inoculation.

***Rooting, hardening and acclimatization:*** The regenerated shoots were transferred onto MS medium supplemented with various concentrations of IBA (0.5–4.5 mg/l) with varying concentrations of NAA (0.5–4.5 mg/l) and a combination of IBA and NAA (0.5–3.5 mg/l) for rooting under an alternative photoperiod scheme (16 h light/8h dark) for 4 to 5 weeks. A total of 17 media combinations (MS+0.5NAA, MS+1.5NAA, MS+2.5NAA, MS+3.5NAA, MS+4.5NAA, MS+0.5IBA, MS+1.5IBA, MS+2.5IBA, MS+3.5IBA, MS+4.5IBA, 0.5IBA+0.5NAA, 0.5IBA+1.5NAA, 0.5IBA+2.5NAA, 0.5IBA+3.5NAA, 0.5NAA+1.5IBA, 0.5NAA+2.5IBA, 0.5NAA+3.5IBA) were tested. Root induction frequencies were recorded at this stage. After 4 to 5 weeks, the plantlets were removed from culture flasks and washed to remove adhering agar from the roots. The plantlets were placed in a small jar containing sterile water for three days and transferred into pots containing soil.

## Results and Discussion

***Callus induction and embryogenesis*:** The present study used mature seed explants for callus induction and embryogenesis. Surface sterilized seeds were inoculated onto a basal MS medium containing different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mg/l) of 2, 4-D for callus induction. Mature seeds were most commonly used explants for obtaining embryogenic callus of range grasses such as *Cenchrus* (Batra and Kumar, 2002; 2003; Kumar and Bhat, 2012; Dwivedi *et al.,* 2016) *Dichanthium* (Gupta *et al.,* 1997; Gupta *et al.*,1998; Bhat *et al.,* 2001; Dalton *et al.,* 2003; Kumar *et al.,* 2005), *Sehima* (Purohit and Kukda, 1995) and *Heteropogon* (Purohit and Kukda, 1996). However, immature embryos and inflorescence were also used as explants for callus induction of graminaceous species (Kackar and Shekhawt, 1991; Yadav *et al.,* 2009). The callus initiation was observed after one week of culture on MS containing 2,4-D (0.5–4.5 mg/l). Purohit and Kukda (1995) also reported the callus initiation on 7$^{th}$ day in Sehima. After three weeks, a big callus (2–4 cm diameter) formation was observed, which was watery and loose. Subculturing improved the growth and texture of the callus. Repeated subculturing of these calli in MS media containing varying concentrations of 2,4-D resulted in the formation of hard and compact embryogenic calli having somatic embryos (Fig 1).

The callus induction frequency was observed to select the best media combination for embryogenic callus induction. The callus induction frequency was observed 40.7 ± 1.7, 51.8 ± 2.3, 61.8 ± 2.8, 62.9 ± 2.3, 71 ± 1.1, 75.5 ± 1.9, 95.8 ± 2.3,

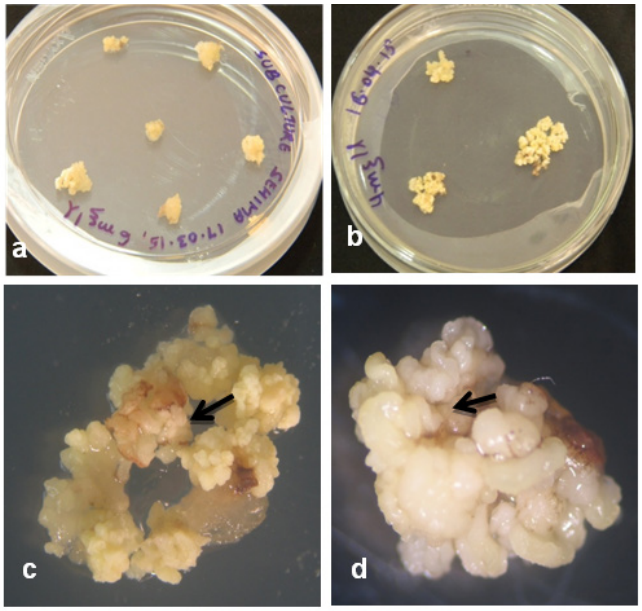


**Fig 1.** *In-vitro* callus induction and somatic embryogenesis from seed explants of *S. nervosum*: (a) callus induction on embryogenic callus induction medium (ECIM) containing 3.5 mg/l 2,4-D after 3 wk; (b) development of somatic embryos on embryogenic callus induction medium containing 3.5 mg/l 2,4-D after 4 wk ; (c) Close up of embryogenic callus with proembryogenic masses (PEM) on embryogenic callus induction medium containing 3.5 mg/l 2,4-D after 4 wk (arrow indicates PEM); (d) somatic embryos

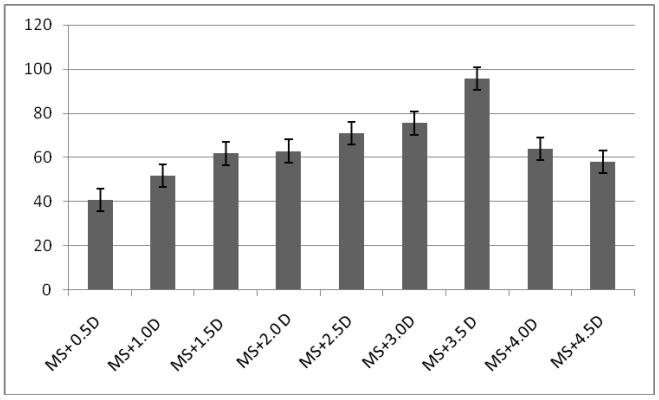


**Fig 2.** Influence of different concentrations of 2, 4-D on embryogenic callus induction from mature seeds of *S. nervosum*

64 ± 2.8 and 58.1 ± 1.7 as in 2,4-D concentration of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mg/l, respectively (Fig 2). The callus induction frequency was enhanced with the increasing concentration of 2, 4-D. The highest callus induction frequency was 95% at 3.5 mg/l 2, 4-D. Overall, 3.5 mg/l 2, 4-D showed the best callus initiation response, suggesting that exogenous auxin levels might influence callus initiation. Analysis of variance showed significant differences among various treatment sets, indicating the role of media combinations in the callus induction frequency. Treatment numbers MS+3.5 D, MS+3.0 D, and MS+2.5 D in decreasing order were the best combinations and significantly better than other treatments. T1 was significantly poor as compared to other treatments. It indicated that for callus induction, MS with 2,4D ranging from 2.5 to 3.5 could be used successfully.

Purohit and Kukda (1995) also showed that 3.0 mg/l 2, 4-D gave the best response to callus induction in Sehima. In range grasses, a better response of 2, 4-D for callus induction was observed in *Cenchrus* (Sankhla and Sankhla, 1989; Kackarand Shekhawat, 1991; Batra and Kumar, 2002; 2003; Yadav *et al.*, 2009; Kumar and Bhat, 2012; Dwivedi *et al.*, 2016) and *Dichanthium* (Gupta *et al.*, 1997; Gupta *et al.*, 1998; Bhat *et al.*, 2001; Dalton *et al.*, 2003; Kumar *et al.*, 2005), *Sehima* (Purohit and Kukda, 1995) and *Heteropogon* (Purohit and Kukda, 1996). Our results were consistent with the previous studies, which also observed differences in callus induction due to various concentrations of 2,4-D.

***Shoot regeneration*:** In Sehima, shoots were regenerated in MS media with different concentrations (0.5–3.0 mg/l) of Kinetin, BAP (0.5-3.0 mg/l) and a combination of Kinetin + BAP (0.5–2.5 mg/l). All the combinations led to shoot regeneration (Fig 3-5). The sudden increase of BAP from 1 to 2 and then to 3 mg/l with a constant level of kinetin led to the germination of somatic embryos with the production of shoots. The highest frequency of shoot regeneration (98%) occurred with 2.5 mg/l kinetin used. Analysis of variance showed that different treatments

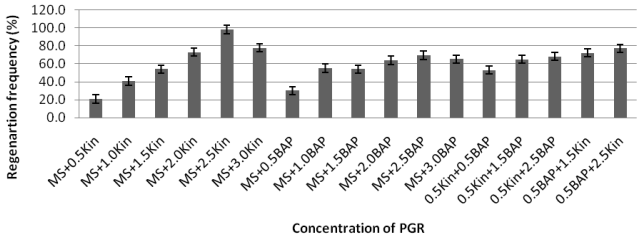


**Fig 3.** Influence of different concentrations of PGR on shoot induction of *S. nervosum*

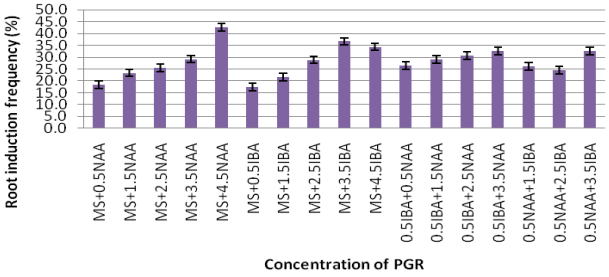


**Fig 4.** Influence of different concentrations of PGR on root induction of *S. nervosum*

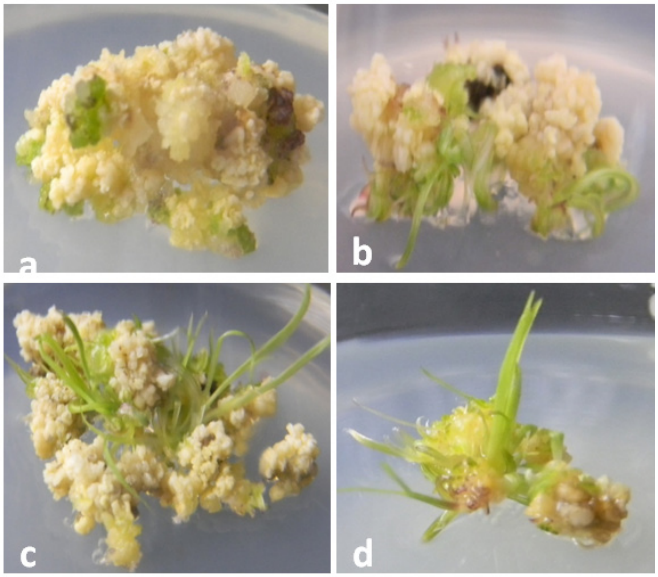


**Fig 5.** *In-vitro* shoot induction from embryogenic callus of *S. nervosum*: (a) Initiation of shoot induction on shoot induction medium (ECIM); (b) development of green shoots; (c) shoot elongation; (d) growth phase of shoots

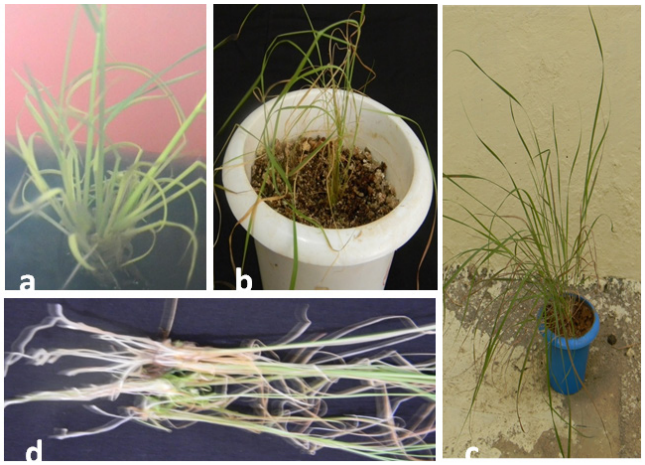


**Fig 6.** *In-vitro* plant regeneration of *S. nervosum*: (a) development of green shoots in soil rite; (b) and (c) plants in pot; (d) whole plants showing well developed root and shoot

of media combinations differed significantly for the induction of shoot. MS+2.5 KiN showed a high shoot induction frequency and was significantly better than other treatments. It was followed by MS+3.0 KIN, 0.5 BAP + 2.5 KIN, and MS+2.0 KIN, which were better than other treatments but were at par statistically among themselves. This was in contrast to an earlier study by Purohit and Kukda (1995), who reported plant regeneration in auxin-free media in Sehima. Our results were similar to those of Batra and Kumar (2002) and Yadav et al. (2009), who also observed shoot regeneration in media containing BAP and Kinetin in Cenchrus.

***Rhizogenesis and hardening*:** Rooting of shoots was successful on media containing various concentrations of IBA (0.5–4.5 mg/l) with varying concentrations of NAA (0.5–4.5 mg/l) and a combination of IBA and NAA (0.5–3.5 mg/l). The maximum root induction frequency occurred with MS + 4.5 mg/l NAA (Fig 4). The maximum number of roots produced per shoot was five. Multiple shoot induction was also observed, possibly due to the higher concentration of cytokinins. Well-rooted plants were transferred to pots and grown at 26 ± 2°C temperature with an alternative photoperiod scheme (16 hours light/8 hours dark) (Fig 6) compared to non-regenerated mother plants. Sankhla and Sankhla (1989) reported that well-developed roots grew profusely on basal MS medium with different cytokinin combinations. Kackar and Shekhawat (1991) and Yadav *et al*. (2009) observed well-developed roots with root hairs in ½ MS, adding 1-naphthalene acetic acid as an auxin.

## Conclusion

The present study successfully achieved as high as 95% callus induction and 98% regeneration in *S. nervosum* for the first time. This information might be necessary in view of generating transgenics in this important crop. Furthermore, this exceptionally high response to callus induction and regeneration in this crop showed its potential as a model crop to undertake biotechnological approaches for perennial range grass improvement.

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