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ORIGINAL ARTICLE

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Induction of Somatic Embryo and Plantlet Regeneration from Immature Inflorescence Culture in Kodo Millet (*Paspalum scrobiculatum* L.) under Salinity Stress Conditions

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Salinity stress is a major abiotic stress factor that affects plant growth, physiological activities and developmental processes. This study involves establishing efficient somatic embryogenesis and plantlet regeneration system using immature inflorescence of kodo millet (*Paspalum scrobiculatum* L. cv. TNAU86) under NaCl-salinity stress conditions. To begin with, the immature inflorescence (0.5cm) of kodo millet was excised from the 40-45 days old field growing plants followed by surface sterilization and inoculation in Murashige and Skoog (MS) medium supplemented with the various concentrations of NaCl (10mM, 25mM, 50mM, 75mM, 100mM, 150mM, and 200mM) salt along with 2,4-Dichlorophenoxyacetic acid (2,4-D) (1.5 mg/L). The results reveal that the maximum mean frequency (89.3±0.3%) of somatic embryogenesis was obtained from embryogenic callus that was growing with medium added with NaCl (50mM) while it was found to be the least mean frequency (12±4.1%) with 150mM of NaCl-salt treatments. Moreover, the highest concentration of NaCl (200mM) salinity was found to be lethal and explants were observed to get gradually necrosed. Later, embryogenic calli showing differentiation of somatic embryos were sub-cultured on basal medium supplemented with 0.5mg/L of 6-Benzylaminopurine (BAP) along with respective concentrations of NaCl-salt for the germination of somatic embryos into plantlets. Significantly, 100mM of NaCl-treatment was proved to show strong inhibitions and thus minimum salt tolerant plantlets regeneration (4±2.21%) was recorded. Further, *in vitro* grown salinity stress tolerant plantlets were transferred to plastic cups and gradually acclimatized under greenhouse conditions.

Key words: Immature inflorescence, Kodo millet, Regeneration, Salinity stress, Somatic Embryogenesis

Millets are C_4 plants found in arid and semi-arid regions (O'Kennedy *et al.*, 2006) having the capacity to fight against diabetic and main source for the calories and proteins. Several millets such as, pearl millet, kodo millet, finger millet, proso millet, little millet and foxtail millet have phenol compounds (Rao *et al.*, 2011), which induce the anti-proliferative property that helps to prevent cancer (Chandrasekara and Shahidi, 2011).

Paspalum scrobiculatum L. is traditionally used to treat diabetes mellitus, the particular reason to increase the serum insulin level significantly and also causes fall in fasting blood glucose (Murthy and Subramanyam, 1989; Jain *et al.*, 2010; Kiran *et al.*, 2014). Also, it provides the gluten free protein; however, it has less protein than other millets. Moreover, it consists of vitamins and minerals, especially B-complex vitamins, B6, niacin and folic acid, Fe, Ca, Mg, K, Zn and also rich in essential amino acids (Ravindran, 1992; Parvathy and Thayumanavan, 1995; Anthony *et al.*, 1996; Gopalan and Shastri, 2009).

India is the largest millet producer in the world. Significantly, in order to create awareness about nutritional food to the people globally; United Nations has declared 2023 as International Year of Millets (IYOM-2023).

Due to the recent climatic changes and environmental stresses, the productivity of the crops was affected. According to Abdel *et al.*, (2003), the salinity and drought stress are the two major abiotic stresses that affect the crop productivity. The environmental stresses like presence of high salinity in the soil affect the crop productivity in terms of quality and also quantity (Vikrant, 2015).

Salinity stress mainly affecting the places in arid and semi-arid areas (Hernández, 2019). The ability of water utilization of plants reduces due to salinity stress and retard the plant growth rate. Also, it changes the metabolism of the plants (Munns, 2002). In order to resolve the difficulties, *in vitro* plant tissue culture has been practiced especially having the capacity of enhancing the tolerance of the plant against abiotic factor like salinity, drought, heavy metal stresses, the major responsibility of reducing the productivity of the crops (Abdel-Qader *et al.*, 2003).

Also, the techniques in *in vitro* tissue culture considered as most efficient method and also having its own values by selection of the valuable crop cultivars. Moreover, *in vitro* propagation of millets helps to achieve the improvements among millets because of growth and productivity was severely affected by environmental stresses. Moreover, it is obligate necessary to build the well-structured establishment of somatic embryogenesis and plantlet regeneration for the recovery of transgenic millet crops (Ceasar and Ignacimuthu, 2009).

Embryogenic callus and somatic embryogenesis from immature tissues have been achieved in different millets. *Paspalum scrobiculatum* has been described by Nayak and Sen (1989) and Vikrant and Rashid (2001), *P. dilatatum* (Akashi and Adachi, 1992), *P. notatum* (Bovo and Mroginski, 1986 and 1989), *P. vaginatum* (Cardona and Duncan, 1997), Likewise, *Eleusine coracana* (George and Eapen, 1990), *Panicum maximum* (Lu and Vasil, 1982) and *Setaria italica* (Vishnoi and Kothari, 1996).

Moreover, it is documented that the immature inflorescence and tissues have been proved to be the best type of explants because of being capable to regenerate the plantlets in high frequency compared to the mature embryos or seeds, especially in recalcitrant crops like monocotyledons (Jha *et al.*, 2009).The objective of the study was to analyze the induction of somatic embryo under salinity stress conditions followed by regeneration of stress tolerant plantlet from immature inflorescence of kodo millet as explant.

MATERIALS AND METHODS

Collection of Plant Material

Seeds of *Paspalum scrobiculatum*L. cv. TNAU86 were collected from Centre of Excellence in Millets, Thiruvannamalai, Tamil Nadu (India). The kodo plants were cultivated in APJ Abdul Kalam Garden, KMGIPSR, Puducherry. The Immature inflorescences (0.5cm) were excised from 40–45-day-old field grown plants and were used as explants.

The explants were surface sterilized using 'tween - 20' followed by 70% (v/v) ethanol for 15 seconds.

Further, explants were then treated with 0.1% $HgCl_2$ about 5 minutes followed by the sterilized distilled water (4-5 times) wash in Laminar Air Flow chamber. The sterilized explants were subjected to nutrient medium further.

Nutrient Medium for Induction of Callus and Somatic Embryogenesis

To regulate the protocol, the immature inflorescence cultured in various concentrations of auxin (2,4-D) along with or without cytokinins (BAP, kinetin, and zeatin). Initially, the explants were cultured on the nutrient medium consisting of MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 2% sucrose (w/v) alongwith different concentrations and combinations of plant growth regulator **(TABLE 1).**

The cultures were incubated at 25±2°C in the dark for callus induction. The cultures were subsequently sub-cultured in respective fresh medium every two weeks routinely.

Nutrient Medium for Plantlets Regeneration

After six weeks, the embryogenic callus was sub-cultured into regeneration media. The MS medium supplemented with different concentrations of phytohormones **(TABLE 2).** These cultures were incubated at 25±2°C with 16/8h (light/dark) photoperiod for 14 days. The germination of somatic embryos and regeneration of plantlets were observed after 10 days of sub-culture.

The regenerated plantlets were gradually acclimatized and transferred to pot containing mixture of soil: sand: vermicompost (1:1:1) in the greenhouse condition.

Salinity Stress Treatments

To determine the effect of salinity stress, the explants were inoculated in MS medium containing 2, 4-D (1.5 mg/L) along with various concentrations (10, 25, 50, 75, 100, 150, and 200mM) of NaCl (v/v) salt solutions. The MS medium supplemented with 2,4-D (1.5 mg/L) and without NaCl is considered as control. The cultures were maintained at optimum temperature (25±2°C) and light condition for 2 weeks interval of time.

Once the callus formation was observed, it subsequently sub-cultured in regular interval of time for somatic embryo induction. The embryogenic calluses were sub-cultured into regeneration medium along with respective concentrations of NaCl **(TABLE 3)** for plantlet germination from somatic embryos. The germinated plantlets were further acclimatized into pot.

STATISTICAL ANALYSIS

Each experiment was repeated three times. The mean percentage of somatic embryogenesis was calculated after 8 weeks (No. of callus showing Somatic embryogenesis / Total no. of Callus X 100). The mean number of shoots regenerated form the somatic embryos were calculated after 20 days of somatic embryo germination. The data were presented as student ‘t’ test to compare its significance.

RESULTS

In order to establish embryogenic callus formation followed by somatic embryogenesis in kodo millet, immature inflorescence explants were treated with various concentrations of auxins and cytokinins were used in present study **(Table 1).**

Callus induction and Somatic Embryogenesis

In basal medium, explants were found to be non-responsive in terms of callus induction even after 10-days of culture initiation. Moreover, such explants were found to be necrosed with slight proliferation in explants tissues **(Fig. 1A)**. In contrast, the explants that were treated with 2,4-D concentration (1.5mg/L) were found to exhibit good amount of nodular and compact callus induction **(Fig. 1C)**.

The frequency of callus induction found in 2,4-D with cytokinins (BAP, kinetin and zeatin) was observed to be relatively less when compared to the frequency of callus induction found in 2,4-D alone supplemented culture **(Table 1)**. Furthermore, the higher concentration (3.0mg/L) of 2,4-D supplemented culture exhibit non-embryogenic callus results with browning of callus in course of time.

Calli that were growing with 2,4-D containing nutrient media were further sub-cultured into same nutrient media after 2 weeks of culture initiation. Small white colored, globular somatic embryos were observed in the embryogenic callus after 1-2 weeks of sub-culture **(Fig. 1D-F).**

The yellowish compact embryogenic callus showing

highest frequency of somatic embryogenesis (87.8±0.5%) was recorded with the explants that were treated with the lowest concentration of 2,4-D (1.5mg/L). However, explants that were treated with combination of 2,4-D (3.0mg/L) with zeatin (0.1mg/L) show the least frequency (31.6±1.6) of callus induction in comparison to the other combination of plant growth regulators **(Table 1)**.

Plantlet Regeneration

The embryogenic callus with mature somatic embryos was sub-cultured into various concentrations of regeneration medium **(Table 2).** The regeneration frequency was found to be maximum at 0.5mg/L of BAP (78.8±0.63%) **(Fig. 2A-B)**, followed by basal medium the germination frequency found to be relatively less (64.9±0.37%). While the germination frequency was found to be the minimum (11.7±0.87%) on the IBA (1.0mg/L) and kinetin (0.5mg/L) combination.

Effects of Salt Stress on Somatic Embryogenesis

The difference between the control as well as the salt treated callus was significant. The culture was observed a primary callus **(FIG.1G)** in lower concentration of NaCl stress (50mM). Later, it formed the embryogenic callus after two weeks of incubation. Perhaps, the salt treated explants show good embryogenic callus and formed the milky white somatic embryos after 15 days of incubation **(Fig.1 J&K)**.

The minimum concentrations (50mM) of NaCl could promote the somatic embryogenesis up to maximum frequency (89.3±0.3%). However, the higher concentration (150mM) of NaCl shows the least mean frequency percentage (12.0±4.1%) for somatic embryogenesis and proved to be little toxic **(Table 3)**. Significantly, further increase in NaCl concentration (200mM) observed that the callus was necrosed **(Fig. 1L)** thus it was proved to be lethal concentration of NaCl for any morphogenic response.

The independent 't'- test to compare the embryogenesis in control and salinity stress treated culture. There was a significant difference between the above study ($t = 7.3$, $p<0.05$). The mean frequency of somatic embryogenesis under salinity condition (50mM) (89.3±0.3%) higher than the mean frequency of somatic embryogenesis in control condition (87.0±0.5%).

Plantlets Regeneration under Salinity Stress

The embryogenic callus with somatic embryos was sub-cultured into nutrient medium with or without 0.5mg/L of BAP along with the respective NaCl concentrations **(Table 4).** The control **(Fig. 2B)** medium shows the highest frequency (78±0.63%) of plantlets regeneration. The plantlet germination from somatic embryos was recorded as the maximum frequency (69.2±0.77%) with 25mM of NaCl **(Fig. 2C&D)** followed by 50mM of NaCl stress (54.6±0.92%) while the least frequency (04.7±2.21%) of plantlet regeneration was obtained with 100mM of NaCl treatment. The *in vitro* plantlet that grown under salinity stress was considered as salt-tolerant and was transferred to plastic cups for further acclimatization process under greenhouse condition **(Fig. 2H).**

Table 1: *Paspalum scrobiculatum* L., effect of auxin either alone or along with cytokinin on callus induction and somatic embryogenesis in immature inflorescence culture.

Concentration of Auxin (mg/L)		Concentration of Cytokinins (mg/L)		Percentage of Embryogenic Callus Mean ± SD	Mean Number of Somatic Embryos/Callus (Mean ± SD)
2, 4- D	1.0	BAP	0	76.5±1.4	48.2±0.9
	1.5			87.8±0.5	58.4±0.2
	2.0			71.3±3.0	43.2±0.3
	3.0			41.7±1.5	14.8±0.9
	1.0		0.5	48.0±1.6	30.3±1.3
	2.0			63.9±9.2	27.9±0.5
	3.0			53.8±2.6	19.4±0.4
	1.0	Kn	0.5	68.3±2.1	34.5±0.9
	2.0			58.1±1.5	25.8±1.3
	3.0			35.3±2.5	9.5±1.7
	1.0	Zn	0.1	65.2±3.5	30.8±0.6
	2.0			57.5±2.0	21.5±0.8
	3.0			31.6±1.6	11.9±0.2

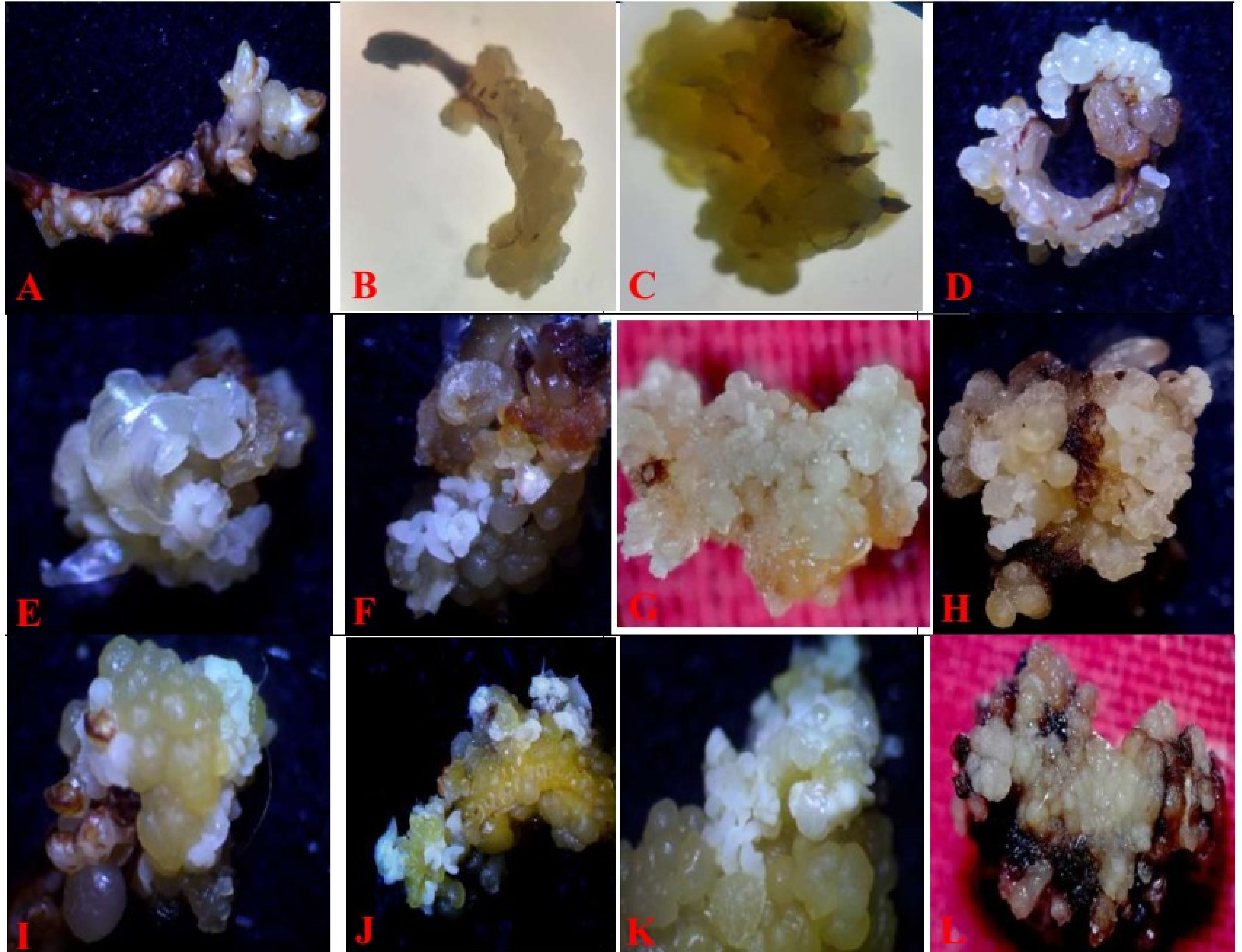

Figure 1. *Paspalum scrobiculatum* L. Immature Inflorescence culture on MS- medium supplemented;

(A) Basal medium; **(B)** callus formation under 1.0 mg/L of 2,4-D; **(C)** callus formation under 1.5mg/L of 2,4-D; **(D)** somatic embryogenesis on 1.0mg/L of 2,4-D; **(E)** somatic embryogenesis on 1.5mg/L of 2,4-D; **(F)** somatic embryogenesis on 2.0mg/L of 2,4-D; **(G)** callus formation on 1.5mg/L 2,4-D with 50mM NaCl; **(H)** callus formation on 1.5mg/L of 2,4-D with 100mM of NaCl; **(I)** somatic embryogenesis on 1.5mg/L of 2,4-D with 25mM of NaCl; **(J-K)** somatic embryogenesis on 1.5mg/L of 2,4-D with 50mM of NaCl; **(L)** callus formation on 1.5mg/L of 2,4-D with 200mM of NaCl.

Table 2: *Paspalum scrobiculatum* L., effect of auxin and cytokinin on regeneration of plantlets in immature inflorescence culture

Concentration of Auxins (mg/L)		Concentration of Cytokinins (mg/L)		Percentage of embryogenic callus showing plantlets regeneration (Mean±SD)	No. of Plantlets regeneration / Embryogenic callus (Mean±SD)
2, 4- D	0	**BAP**	0	64.9±0.37	22.8±0.79
	0		0.5	78.8±0.63	31.3±0.20
	0.5			37.5±0.94	19.5±1.53
	1.0			23.3±0.97	9.1±0.71
	1.5			15.1±1.40	8.3±1.63
	0	**Kn**	0.5	29.4±1.81	11.2±1.01
	0.5			14.5±0.32	5.3±0.80
	1.0			20.2±0.97	3.5±1.41
IBA	0.5	**BAP**	0.5	24.1±0.60	9.9±0.81
	1.0			18.6±0.53	5.9±0.93
	0.5	**Kn**	0.5	13.4±0.72	7.4±0.62
	1.0			11.7±0.87	6.3±0.87

Table 3: *Paspalum scrobiculatum* L., effect of NaCl-salinity stress on somatic embryogenesis in immature inflorescence culture

Concentration of NaCl (mM)	Concentration of 2,4-D (mg/L)	Percentage of Somatic Embryogenesis (Mean±SD)	Mean Number of Somatic Embryos/Callus (Mean ± SD)
0	1.5	87.0±0.5	58.1±0.20
10	1.5	83.9±1.6	49.7±0.41
25	1.5	85.3±0.4	52.4±0.76
50	1.5	89.3±0.3	64.7±0.82
75	1.5	68.9±0.2	48.3±0.82
100	1.5	31.3±0.5	16.3±0.98
150	1.5	12.0±4.1	08.6±1.23
200	1.5	0	0

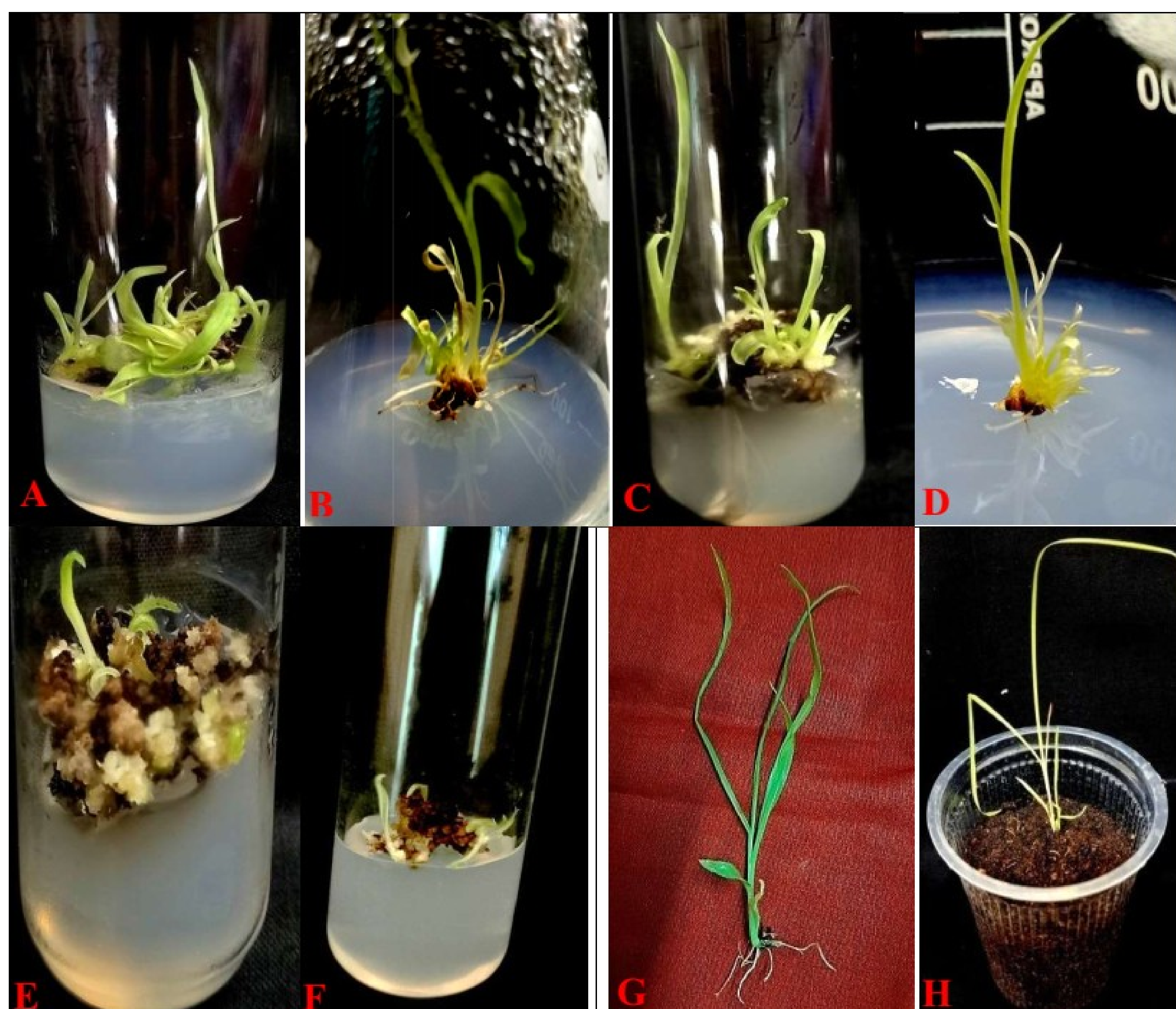

Figure 2. Salinity Stress-*Paspalum scrobiculatum* L. Immature Inflorescence culture showing plantlet regeneration; **(A-B)** Control (0.5mg/L of BAP); **(C-D)** Plantlets regeneration on 0.5mg/L of BAP with 25mM NaCl; **(E)** Plantlets regeneration on 0.5mg/L of BAP with 50mM of NaCl; **(F)** Plantlets regeneration on 0.5mg/L of BAP with 100mM of NaCl **(G)** Salinity tolerant plantlet; **(H)** Potted plantlet.

Table 4: *Paspalum scrobiculatum* L., effect of NaCl-salinity stress on plantlets regeneration in immature inflorescence culture

Concentration of NaCl (mM)	Concentration of BAP (mg/L)	Percentage of embryogenic callus showing plantlets regeneration (Mean%±SD)	No. of Plantlets Regeneration/ Embryogenic Callus (Mean±SD)
0	0.5	78.8±0.63	31.9±0.20
25	0.5	69.2±0.77	23.6±0.78
50	0.5	54.6±0.92	11.6±1.58
100	0.5	04.7±2.21	1.3±1.87

DISCUSSION

Abiotic stress in general and salinity in particular has been proved to be a big challenge for our agriculture yields. However, one of the important strategies has been adopted to overcome salinity is to exploit the *in vitro* regeneration procedures under salinity stress conditions to identify and achieve salinity tolerant genotype that may sustain a reasonable yield on salt affected soils (Ashraf *et al.*, 2006).

Establishment of Somatic Embryogenesis in Millets

The immature tissues, such as immature and mature zygotic embryos (Vikrant and Rashid, 2002a), immature inflorescence (Nayak and Sen, 1989; Vikrant and Rashid, 2001; Kaur and Kothari, 2004), leaves and roots (Kaur and Kothari, 2003; Hoque and Mansfield, 2004), mesocotyl and leaf segments (Vikrant and Rashid, 2003) of kodo millets were used as explants in order to establish the induction of somatic embryogenesis and plantlet regeneration. According to the literature, the immature inflorescence shows greatest embryogenesis and regenerative capacity rather than the other explants such as, mature seeds and shoots tips (Jha *et al.*, 2009).

The present study involves the both somatic embryogenesis and regeneration from immature inflorescence as explants in *Paspalum scrobiculatum* L. to induce the salinity stress tolerant plantlets in efficient way in major cereals and millet crops. 2,4-D was used in order to induce callus on millets and other crops (Mohanty *et al.*, 1985; Chandra and Kothari, 1995; Kothari-chajer *et al.*, 2008; Sonia Plaza-Wüthrich and Zerihun Tadele, 2012).

To induce embryogenic callus, the present study involves auxins (2,4-D) tested alone or with combinations of BAP, kn and zeatin. The medium containing 2,4-D alone observed that increased frequency percentage of somatic embryogenesis (1.5mg/L). Recently, Kothari-chajer *et al.*, 2008 used 8.6µM 2,4-D alone in the micronutrient-modified MS medium for somatic embryogenesis of the kodo millet genotype GUPK3.

It has been reported that the higher concentrations of auxin found to inhibit the induction of embryogenic calli. In contrast, the lower concentration of auxin is proved to be effective in cereals (Lu *et al.*, 1983; Bi *et al.*, 2007) and other millets like, sorghum (Amali *et al.*, 2014).

However, in present study, 2,4-D alone could be proved more effective in terms of induction of somatic embryos than the combination of cytokinin with 2,4-D. Moreover, in contrast, the combination of 2,4-D along with BA, kinetin and zeatin increases the percentage of embryogenic callus induction in *Eleusine coracana* (Ceasar and Ignacimuthu, 2008), *Sorghum bicolor* (Wernicke and Brettell, 1980; Belide *et al.*, 2017; Espinoza-Sánchez *et al.*, 2018).

Germination of Somatic Embryo and Plantlets Regeneration

Historically, a direct organogenesis from immature inflorescence has been observed in millets (Kavi Kishore *et al.*, 1992). However, the combinations of BA and NAA were used to promote the regeneration from somatic embryos in *Paspalum scrobiculatum* L. (Nayak and Sen, 1989; Arokiyasamy*et al.*, 2001; Kaur and Kothari, 2004), *P. vaginatum* (Cardona and Duncan, 1997).

Moreover, an efficient regeneration protocol from shoot apex (Arokiyasamy *et al.*, 2001) and immature inflorescence (Vikrant and Rashid, 2001; Kothari-chajer *et al.*, 2008) could be possible by modifying medium

micronutrient in *P. scrobiculatum.* Apart from auxins, the cytokinins (kinetin and BA) also have been used for plantlet regeneration from somatic embryos in pearl millet (Mythili *et al.*, 2001; Srivastav and Kothari, 2002), finger millet (Ceasar and Ignacimuthu, 2008) and foxtail millet (Xu *et al.*, 1984).

Hence, in the present study, the BAP (0.5mg/L) was proved to be helpful to promote the plantlet germination frequency (78.8±0.63%) in comparison with kinetin (0.5mg/L supplemented medium) (29.4±1.81%). Whereas, the combination of kinetin (0.5mg/L) with IBA (1.0mg/L) observed to be least germination frequency (11.7±0.87%) of somatic embryos. The plantlet regeneration from embrogenic callus was highly boosted by cytokinins than auxins (Girgi *et al.*, 2002) in finger millet (Sankhla *et al.*, 1992; Yemets *et al.*, 2003; Latha *et al.*, 2005; Ceasar and Ignacimuthu, 2008; Nethra *et al.*, 2009), kodo millet (Ceasar and Ignacimuthu, 2010) and pearl millet (Mythili *et al.*, 1997; Goldman *et al.*, 2003; Satyavathi *et al.*, 2016).

Somatic Embryogenesis under Salinity Stress

The present study reveals that, the frequency of callus induction as well the somatic embryogenesis was found to be higher (89.3±0.3%) when it is exposed to saline stress (50mM) as it compared to control (87.0±0.5%). In literature, the increase in saline concentration leads to the reduction of callus formation as well as somatic embryogenesis (Vikrant, 2015).

There were many reports stated that, some genotypes were producing high germination frequency, high yield and biomass, when they treated with salt condition (Ashraf *et al.*, 2006; Krishnamurthy *et al.*, 2007). In some cases, the germination rate found to be reduced because, the reduction in water potential; hence, it affects the water absorption due to, toxic effect of saline condition (Jamil *et al.*, 2006). The increase in NaCl salt concentration (150mM) decreases the callus induction and affects the somatic embryo formation in 2, 4-D supplemented medium (12.0±4.1%).

Plantlet regeneration under Salt Stress

The establishment of *in vitro* regeneration from somatic embryogenesis under saline stress condition has been reported in many plants. The plantlet germination was found to be inhibited while the increase in the concentrations of NaCl treatments. The control medium (0.5mg/L of BAP) shows the highest frequency of somatic embryo germination (78.8±0.63%). The lowest concentration of NaCl (25mM) shows moreover, mention equally high number of plantlets regeneration (69.2±0.77%) as compared to control. In contrast, the maximum shoot length was produced by some genotypes when they were exposed to NaCl stress. Even they show high mean frequency of shoot length under salinity stress condition for saline tolerant genotypes (Hakim *et al.*, 2010; Bashir *et al.*, 2011).

The rate of somatic embryo germination found to be reduced in salt-tolerant cultivar in finger millet (Mukami *et al.*, 2020). Likewise, increase in NaCl concentration (50mM and 100mM) the plantlet germination frequency found to be deceasing (54.6±0.92% and 4.7±2.21%) respectively. The higher concentration (150mM) of NaCl proves to be the lethal for plantlets regeneration.

CONCLUSION

The present study produces the protocol for efficient somatic embryogenesis and plantlet regeneration from the explant immature inflorescence of kodo millet under salinity stress. Interestingly, NaCl (50mM) proves to support somatic embryogenesis instead of inhibiting the process, however, in contrast the same concentration of NaCl (50mM) was found to be inhibitory for the plantlet germination. Also, the plantlet withstands the saline condition when it was acclimatized. This study further could be useful in production of genetic transformation of millet crops in order to withstand the salinity stress and produce higher yields.

Authors Contribution

The first author of the manuscript has carried out the experimental part of the study, compilation of the statistical data and the manuscript preparation. Second author has designed the experimental set up and also edited the manuscript into presentable format.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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