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IMPROVEMENT OF EMBRYOGENESIS AND REGENERATION BY AIR DESICCATION IN MAIZE (*ZEA MAYS* L.)

SELIM MORSHED, ABU BAKER SIDDIQUE AND S.M. SHAHINUL ISLAM*

Plant Genetic Engineering Lab., Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh
**Corresponding author e-mail: shahin_ibsc@ru.ac.bd*

Abstract

Calli derived from mature embryos of four maize varieties *viz.* Mohar, Khoi bhutta, Barnali and Shuvra were cultured in three basal media for regeneration (MS, N6 and 6N1) which individually supplemented with four hormonal combinations e.g. H_1 = BAP 0.5 mg/l + IAA 0.0 mg/l, H_2 = BAP 1.0 mg/l + IAA 0.5 mg/l, H_3 = BAP 1.5 mg/l + IAA 1.0 mg/l and H_4 = BAP 2.0 mg/l + IAA 1.5 mg/l. The highest frequency of regeneration was found with MS + H_2 (41.35%) in Mohar, while the lowest was 17.37% in 6N1 + H_1 for Barnali. To enhance the capability of regeneration, calli were pretreated by ten groups (6, 12, 18, 24, 30, 36, 42, 48, 54 and 60 h) of desiccation periods. The degrees of desiccation of pretreated calli were determined; and it was ranged as 6.23 to 40.52% where Khoi bhutta showed the maximum value at 60 h desiccation. The callus of Mohar exhibited the highest frequency of regeneration (75.24%) which desiccated for 48 h; and it was around 2 fold higher than the control. The variety Khoi bhutta showed the lowest efficiency (31.80%) when the callus was desiccated for 6 h. All the varieties performed their maximum regeneration at different periods, where 36, 30 and 42 h desiccation were optimal for Barnali (67.23%), Khoi bhutta (68.03%) and Shuvra (73.98%) accordingly. Analysis of variance (ANOVA) showed significant effect of maize genotype and periods of partial air desiccation to enhance regeneration at $p<0.05$ level.

Key words: Air desiccation, Pre-treatments, Regeneration, Callus induction, *Zea mays* L.

Introduction

As the consequence of climate change, maize an important crop plant is to face some abiotic stress conditions, such as drought, salinity, heat, cold etc. Hence, improvement of maize genotypes is the demand of the time being like some other major cereal crops (e.g. rice, wheat and barley). It could be done successfully by using the techniques of biotechnology as well as genetic transformation; while high frequency of regeneration is the prior requirement to be succeeded. Therefore, strategies to improve *In vitro* regeneration frequency have been emphasized and are steadily evolving (Huang & Wei, 2004; Che *et al*., 2006). Armstrong (1994) mentioned that generally *In vitro* culture and regeneration are difficult for important crops including maize. Efficient plant regeneration remains limiting factors for most elite lines of maize (Deng *et al*., 2009). However, by applying desiccation pretreatment frequency of plant regeneration has been enhanced in rice (Saharan *et al*., 2004; Siddique *et al*., 2014), wheat (Carman, 1988), soybean (Hammatt & Davey, 1987), grape-vine (Gray, 1987), spruce (Roberts *et al*., 1991) and cassava (Mathews *et al*., 1993). Whereas, regeneration efficiency as well as *In vitro* culture is affected by the genotype including growth regulators and culture media, were reported in many plants (Islam, 2010; Alam *et al*., 2012; Silva *et al*., 2014). So far as we know, using Bangladeshi maize variety, reports on the effect of desiccation to plant regeneration are not available. Therefore, present study has been undertaken to evaluate the regeneration efficiency of maize pre-treated abiotic stress as air desiccation to targeted explants.

Materials and Methods

Plant materials: Mature seeds of four maize varieties *viz*., Barnali, Mohar, Khoi bhutta and Shuvra were collected from Bangladesh Agriculture Research Institute (BARI), Gazipur, Bangladesh. For cultivation, seeds were grown in the research field of Institute of Biological Sciences, University of Rajshahi, Bangladesh. As explant mature cobs were used when its age was around 15 - 16 days that collected from the research field in 2014.

Sterilization, callus induction and plant regeneration: Mature seeds were surface sterilized with 70% (v/v) ethanol for two minutes. Then treated by sodium hypochlorite (NaOCl) for five minutes and rinsed with sterile distilled water (SDW) for 4 - 5 times and subsequently sterilized with 0.1% (v/v) mercuric chloride ($HgCl_2$). After washing with SDW, callus was induced following the protocol of Morshed *et al*. (2014). For regeneration, three basal media i.e. N6 (Chu, 1978), MS (Murashige & Skoog, 1962) and 6N1 (Genovesi, 1990) were used; and each medium individually supplemented with four hormonal combinations H_1, H_2, H_3 and H_4 as shown in Table 1. Petri dishes were incubated in growth chamber under 16/8 h light condition at 25 ± 2°C.

Application of partial air desiccation: The calli ages of four weeks were pretreated with ten (10) desiccation periods (6, 12, 18, 24, 30, 36, 42, 48, 54 & 60 h) and without desiccation (0 h) was considered as control. Callus of vigorous growth was placed into empty petri dishes containing sterile Whatman 1 filter papers followed by the standard protocol of Saharan *et al*. (2004). The petri dishes were sealed with parafilm and incubated in culture room at 27 ± 1°C in dark. Degrees of desiccation of pretreated calli were determined by following formula (Deng *et al*., 2009).

$$\text{Degree of desiccation} = \frac{\text{Weight of fresh calli} - \text{Weight of desiccated calli}}{\text{Weight of fresh calli}} \times 100$$

Desiccated calli were transferred to MS + H_2 for plant regeneration at 16/8 h photoperiod; and regenerated plants with well roots were transferred to pot after acclimatization and hardening for field culture.

Statistical analysis of data: The average or mean values were computed from five replications with standard error (SE). Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were done by SPSS17.0 software. The seed derived calli without pretreatment were considered as control.

Results

Using undesiccated calli, regeneration performance of the studied varieties was tested in three basal media with four types of hormonal combinations (H_1 - H_4); and the results were different significantly. The variety Mohar gave maximum regeneration (41.35%) in MS + H_2 and the minimum (17.37%) was in 6N1 + H_1 for Barnali (Table 1). All the varieties showed better performance in MS than N6 and 6N1 medium. Hence, the effect of partial desiccation was examined by culturing the desiccated calli in MS to enhance regeneration.

Calli were desiccated by ten groups of desiccation periods; and the highest degree of desiccation was found for Khoi bhutta (40.52%) at 60 h; and lowest was for Mohar (35.18%) with same desiccation period (Table 2). In this experiment, lesser degrees of desiccations were appeared at lower periods, and grater degrees were found at higher periods of desiccation. The morphological features of desiccated calli were different in color (Fig. 1, a-c). Analysis of variance (ANOVA) showed a significant difference among the varieties and also in the periods of desiccation.

Effect of partial desiccation to plant regeneration was examined and the results were presented in Table 3. Among the varieties, Mohar showed the highest performance with 75.24% regeneration when the calli was desiccated for 48 h which was around 2 fold higher than the control. The varieties Barnali, Khoi bhutta and Shuvra gave maximum 67.23, 68.03 and 73.98% regeneration at 36, 30 and 42 h desiccation periods respectively. Accordingly, the values (pre-treatment by desiccation) were 1.88, 2.25 and 1.94 folds higher than the control (Fig. 2, f). The calli of Barnali produced the lowest frequency of plant regeneration (29.32%) when it was desiccated for 60 h.

Discussion

Without desiccation pretreatment to the calli, regeneration efficiency of the varieties were ranged as 17.37-36.41%, 29.68-41.35%, 17.47-30.79% and 28.11-40.56% for Barnali, Mohar, Khoi bhutta and Shuvra respectively (Table 1). The varieties were different significantly while the efficiency of regeneration was poor. In some previous reports lower efficiency was recorded in maize from untreated calli (Dhillon & Gosal, 2012; Jia *et al*., 2008). Abebe *et al*. (2008) reported maximum 21% regeneration and mentioned that due to recalcitrance in nature, tropical maize genotypes responded at low rate. Our findings agreed well with the reports and claimed that it's not enough to be succeeded in advance biotechnological research like genetic transformation of maize. Pathi *et al*. (2013) mentioned that 35 - 90% regeneration for Indian local ecotype (HQPM-1) of maize. Present study argued to the report and stated that it might be happened due to different genotype along with hormonal combinations. Such effects were described in several plants e.g. barley (Haque & Islam, 2014), *Taxus wallichiana* (Hussain *et al*., 2013), maize (Morshed *et al*., 2014), rice (Saharan *et al*., 2004). However, overall average values of regeneration from undedicated calli expresses that Mohar carried higher potential to regenerate plants along with MS + H_2 was suitable for *In vitro* culture of maize (Fig. 2a-c).

To enhance the capability of plant regeneration, partial air desiccation pretreatment was applied and desiccated calli at optimal degree were eligible to regenerate with higher frequency. It influenced plant regeneration and produced significant higher values (Table 3). All the varieties exhibited their maximum performance at different periods of desiccation. Hence, it could be suggested that pretreatment with partial desiccation with an optimal level for specific variety was a considerable factor to gain higher regeneration. Deng *et al*. (2009) reported that 1.5 - 2.1 fold higher regeneration after 48 h desiccation compared to the non-desiccated calli, and noticed the effect of desiccation on shoot regeneration, this increase was occurred mainly during the early phase of induction. Rance *et al*. (1994) reported that 2 - 4 folds higher regeneration from 3 h desiccated calli than control in rice. Compare to undesiccated calli, 2 and 5 folds higher regeneration was recorded from 48 and 72 h desiccation in rice genotypes of MR220 and MR232 respectively (Makerly *et al*., 2012). Approximately similar increased regeneration was recorded in rice (Biswas & Mandal *et al*., 2007; Saharan *et al*., 2004) and sugarcane (Kaur & Gosal, 2009). In date palm cultivar 3 and 4 h partial desiccation reduced fresh weight of calli and stimulated calli growth globularization as well as embryo formation (Ibrahim *et al*., 2012).

In our study results agreed with some previous findings while in some cases it argues with lesser enhancement of regeneration. Due to genotypic effect, our results might differ with them, and such statements were reported by several authors (Alam *et al*., 2012; Chand & Sahrawat, 2001; Islam & Tuteja, 2012). However, it could be stated that partial air desiccation had a significant effect to enhance the rate of plant regeneration in studied maize varieties. The phenomena could be dependent on water content (WC) in the cells of the calli. Callus might contain excess water while they need to desiccate that they were dehydrated at optimum level. A suitable dehydration as well as desiccation promoted the calli to exhibit the maximum efficiency. Siddique *et al*. (2014) reported that the degrees of water loss differ against same desiccation period in different genotype, and an optimal level of partial air desiccation could be beneficial to plant regeneration. They added that for desiccation, dehydrates the cells sap; as a result the osmotic potential of the cells of calli was increased which might play a positive role. However, our investigation describes the similar possible causes including genetic variability and degree of desiccation for enhancing the potentiality of the calli to regenerate plants. It was previously reported that loss of more than 20 - 50% water content of the cells is been lethal to most of the higher plants (Kranner *et al*., 2002). Such report developed a hypothesis as over desiccation might be harmful to existence of calli. Our findings agreed well with the findings, because of lower frequencies at top desiccation period. In this study, all the maize varieties showed lowest number of regeneration at the top level of desiccation (60 h) except Khoi bhutta (Table 3). However, analysis of variance (ANOVA) expressed the significant effect of partial air desiccation to enhance plant regeneration in studied maize varieties.

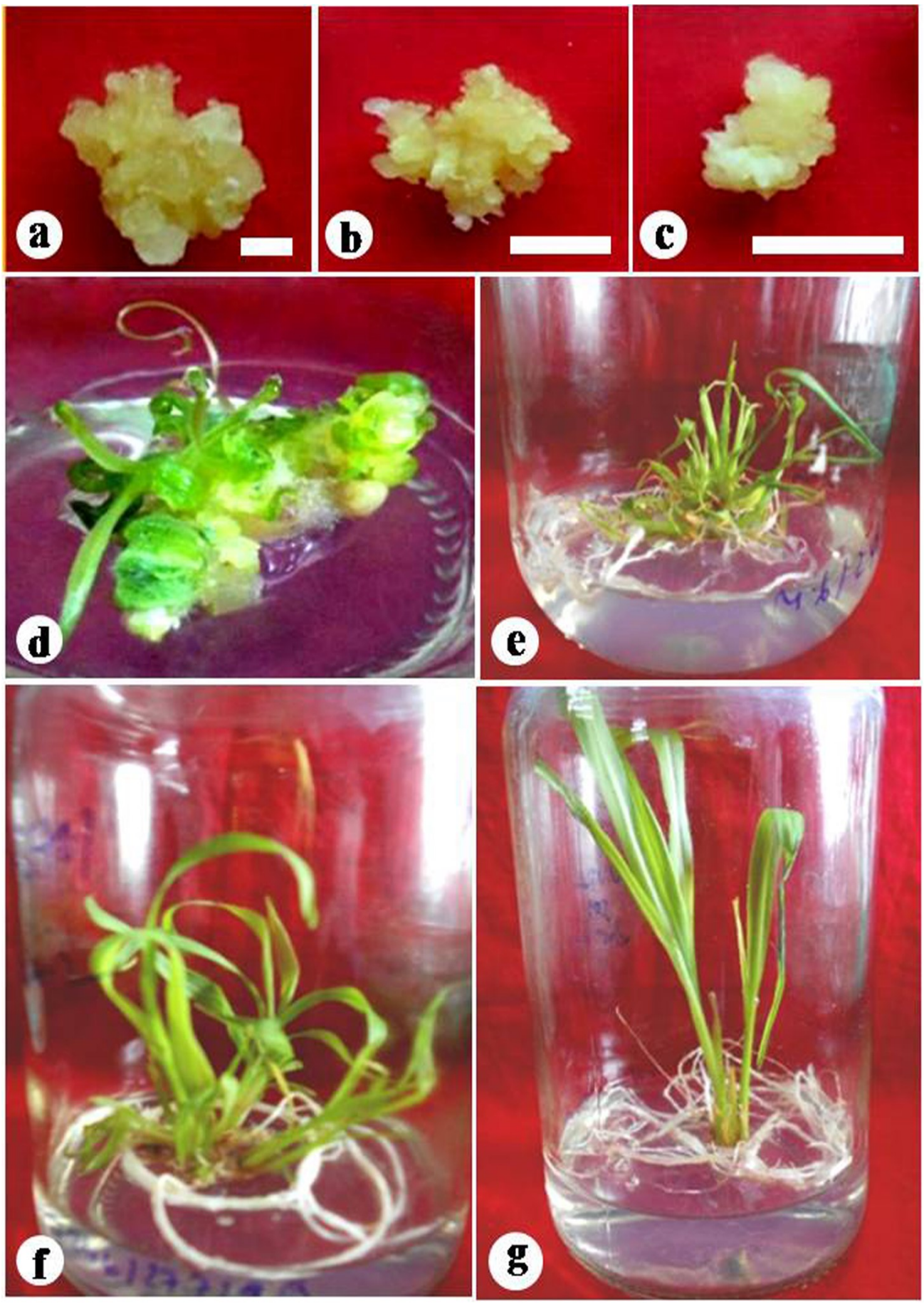

Fig. 1. (a-c) Morphology of desiccated calli (white bars indicate the approximate degree of desiccation). (a) 12 h, (b) 36 h and (c) 72 h desiccation; (d) Embryogenic callus and regenerated shoot, (e) Regenerated plantlet, (f) Well rooted plant, (g) Acclimatized plant.

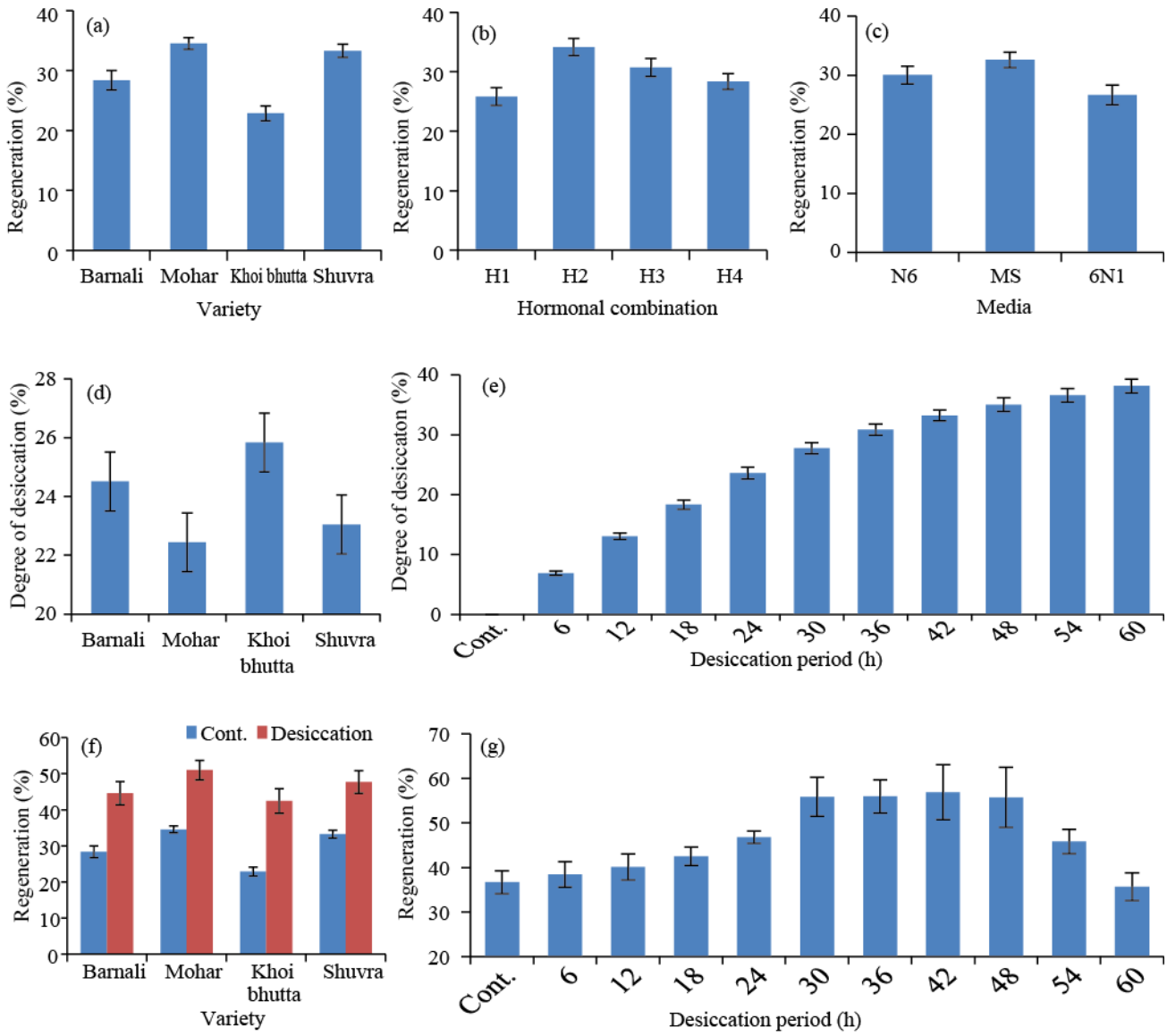

Fig. 2. Overall mean values of various parameters (a-g). Effect of variety, hormone and media to regeneration from un-desiccated calli (a-c); degree of desiccation against variety and desiccation periods (d-e) and regeneration performance form desiccated calli (f-g).

Table 1. Effect of three basal media and four hormonal combinations to plant regeneration from undesiccated calli (% ± SE).

Basal media	Hormonal combination	Variety			
		Barnali	Mohar	Khoi bhutta	Shuvra
N6	H_1	23.27 ± 1.46d	30.86 ± 1.79ef	18.82 ± 1.01de	28.11 ± 1.27f
	H_2	34.81 ± 0.54ab	38.19 ± 0.93ab	26.31 ± 1.30b	36.77 ± 0.76b
	H_3	29.62 ± 1.39c	36.55 ± 1.28bc	24.35 ± 0.73bc	34.98 ± 1.84bc
	H_4	30.93 ± 0.44c	35.39 ± 1.92bcd	20.37 ± 1.80de	31.19 ± 0.91def
MS	H_1	29.64 ± 0.85c	33.63 ± 0.65cde	20.71 ± 0.59de	30.67 ± 0.97ef
	H_2	36.41 ± 1.30a	41.35 ± 0.86a	30.79 ± 1.38a	40.56 ± 1.25a
	H_3	32.51 ± 0.59bc	37.34 ± 1.04bc	29.78 ± 0.92a	36.35 ± 1.22b
	H_4	30.11 ± 1.10c	31.9 ± 1.07def	25.93 ± 1.33b	34.28 ± 0.67bcd
6N1	H_1	17.37 ± 1.07e	29.68 ± 0.48f	17.47 ± 0.83e	29.70 ± 0.69ef
	H_2	31.63 ± 0.98c	35.44 ± 0.96bcd	21.20 ± 0.92cd	36.05 ± 0.79b
	H_3	23.17 ± 0.95d	33.93 ± 1.09cde	17.91 ± 0.97de	32.45 ± 0.90cde
	H_4	20.93 ± 0.91d	30.38 ± 1.01ef	20.73 ± 0.85de	28.35 ± 0.97f
	F-value	33.188	9.163	16.621	12.927
	P-value	0.000	0.000	0.000	0.000

H_1 = BAP 0.5 mg/l + IAA 0.0 mg/l, H_2 = BAP 1.0 mg/l + IAA 0.5 mg/l, H_3 = BAP 1.5 mg/l + IAA 1.0 mg/l, H_4 = BAP 2.0 mg/l + IAA 1.5 mg/l and 2.0% sucrose was constant. In each column the mean values followed by same letter (s) are not significantly different at $p<0.05$ according to DMRT

Table 2. Degree of desiccation of pretreated calli in different desiccation periods (% ± SE).

Desiccation period (h)	Variety			
	Barnali	Mohar	Khoi bhutta	Shuvra
Cont.	0.00 ± 0.00i	0.00 ± 0.00h	0.00 ± 0.00i	0.00 ± 0.00i
6	7.13 ± 0.94h	6.32 ± 0.96g	7.86 ± 1.07h	6.23 ± 1.01h
12	13.64 ± 0.80g	12.34 ± 0.79f	14.25 ± 1.30g	12.04 ± 0.69g
18	18.45 ± 0.71f	17.25 ± 0.57e	20.42 ± 0.87f	17.24 ± 0.62f
24	23.61 ± 0.98e	22.34 ± 1.16d	26.34 ± 0.84e	22.15 ± 1.18e
30	27.33 ± 0.73de	26.86 ± 0.60c	30.51 ± 1.00d	26.35 ± 0.82de
36	31.26 ± 0.83cd	29.52 ± 1.04bc	33.42 ± 0.62cd	29.35 ± 1.26cd
42	34.21 ± 1.18bc	31.25 ± 1.20ab	35.26 ± 0.71bc	32.15 ± 0.78bc
48	36.42 ± 0.85ab	32.21 ± 0.75ab	37.26 ± 1.02abc	34.21 ± 0.86ab
54	38.11 ± 0.86ab	33.56 ± 0.72ab	38.42 ± 0.86ab	36.32 ± 1.28ab
60	39.42 ± 1.04a	35.18 ± 0.95a	40.52 ± 1.04a	37.51 ± 0.91a
F-value	250.014	196.502	263.975	196.207
p-value	0.000	0.000	0.000	0.000

Cont. = Control (without desiccation). Used calli were induced on N6 + 1.0 mg/l BAP + 0.5 mg/l IAA + 2.87 mg/l L-proline + 100 mg/l casein hydrolysate + 10 mg/l $AgNO_3$ + 2.0% sucrose. In each column the mean values followed by same letter (s) are not significantly different at $p<0.05$ according to DMRT

Table 3. Effect of desiccation to plant regeneration of four maize varieties (% ± SE).

Desiccation period (h)	Variety			
	Barnali	Mohar	Khoi bhutta	Shuvra
Cont.	35.83 ± 0.67e	42.56 ± 0.98fg	30.19 ± 1.02g	38.16 ± 0.97gh
6	36.29 ± 0.70e	44.82 ± 0.66efg	31.80 ± 0.81g	40.86 ± 1.15fg
12	38.44 ± 1.16de	46.85 ± 1.12defg	33.09 ± 1.00fg	42.15 ± 1.01efg
18	42.58 ± 1.90d	47.20 ± 1.05cdef	37.21 ± 0.77ef	43.11 ± 1.14ef
24	43.27 ± 1.11d	48.71 ± 1.03cdef	49.17 ± 1.59bc	46.05 ± 0.71de
30	56.23 ± 0.98b	49.92 ± 1.37cde	68.03 ± 0.63a	49.36 ± 1.06cd
36	67.23 ± 0.68a	51.97 ± 0.95bcd	52.69 ± 1.08b	51.93 ± 0.91bc
42	50.51 ± 1.45c	57.10 ± 1.37b	46.04 ± 0.49c	73.98 ± 0.67a
48	48.66 ± 0.62c	75.24 ± 0.93a	45.00 ± 1.30cd	54.02 ± 1.00b
54	42.16 ± 0.71d	52.32 ± 1.07bc	40.63 ± 1.09de	48.33 ± 0.72cd
60	29.32 ± 0.94f	44.04 ± 1.67fg	33.14 ± 1.78fg	36.24 ± 0.69h
F-value	101.820	65.054	108.412	122.661
p-value	0.000	0.000	0.000	0.000

Medium MS + BAP 1.0 mg/l + IAA 0.5 mg/l was constant. In each column the mean values followed by same letter (s) are not significantly different at $p<0.05$ according to DMRT

Conclusion

Partial air desiccation pre-treated as abiotic stress that affected plant regeneration positively and enhanced the capability to produce *In vitro* maize plant. In this investigation the variety Mohar exhibited the highest efficiency to regenerate plants after applying 48 h desiccation stress to the calli. Other varieties performed their maximum regeneration at different optimal levels of desiccation. To improve maize genotypes, air desiccation might be considered as an effective tool for advance biotechnological study which provides increased regeneration.

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