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Effect of Desiccation and Chilling Treatment on Somatic Embryo Development and Germination in Rough Lemon (*Citrus jambhiri* Lush)

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Author's contribution

The author BS solely performed this research.

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ABSTRACT

The goal of this study was to evaluate the effect of desiccation and chilling treatments on somatic embryogenesis of rough lemon (*Citrus jambhiri* Lush.). Styles were cultured on seven culture media (MS I-MS VII) containing Benzylaminopurine (BAP), Kinetin (KN) and Malt Extract for cell proliferation and somatic embryo development. Cell proliferation was maximum on MS IV media but maximum cultures showing somatic embryogenesis (52.08 %) was observed on MS VII media. Embryogenic callus proliferated on MS VII media was subjected to desiccation and chilling treatment for 24, 48, 72 and 96 hours. Embryogenic callus desiccated for 24 and 48 hours in sterile petriplates showed 58.33 and 56.94 % somatic embryogenesis respectively as compared to undesiccated callus (51.98%). Average number of cotyledonary embryos (6.80/culture) in embryogenic cultures from desiccated callus (48 hrs) was more as compared to untreated callus (2.26/culture). There was significantly less number of abnormal embryos (0.60-0.53/culture) in desiccated callus for 48, 72 and 96 hours as compared to untreated callus (7.20/culture). Chilling treatment also improves the average number of cotyledonary embryos and reduces the abnormal development of embryos. Among all the treatments desiccation of embryogenic callus for 48 hrs proved beneficial for improvement of somatic embryo development and germination.

Keywords: Citrus; embryogenic callus; desiccation, chilling; somatic embryogenesis.

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ABBREVIATIONS

BAP-Benzylaminopurine, KN-Kinetin, MS-Murashige and Skoog, 2,4-D-2,4 Dichlorophenoxy -acetic acid, ME – Malt Extract.

1. INTRODUCTION

The genus *Citrus* consists of a number of species of horticultural importance which form an important fruit crop worldwide. Rough lemon (*Citrus jambhiri* Lush.) is the most commonly used rootstock in North Indian states like Punjab, Haryana, Rajasthan and Uttar Pradesh for different citrus cultivars [1]. It develops a deep rooting system, produces heavy yields and gives a long life to scion grafted on it. Rough lemon plants for seed collection were not available in sufficient quantities in Punjab and seeds of this rootstock lose their viability after few days of collection [2]. Rough lemon seedlings were mainly used by nurserymen for grafting of scion of different citrus species. It was also used in plant tissue culture for micrografting of scion for production of virus free citrus plants [3].

Biotechnological techniques such as protoplast fusion and genetic engineering approaches are looked as a valuable strategy for improvement of this rootstock. Production of callus and its subsequent regeneration are the prime steps in citrus to be manipulated by biotechnological means and to exploit somaclonal variations [4]. During plant regeneration from callus, somatic embryo is an intermediate stage between the undifferentiated cells and plantlets. A more efficient protocol of somatic embryogenesis is required to promote and expedite the regeneration of plantlets for this rootstock. Moreover, somatic embryos have a potential for long term storage through cryopreservation [5]. There are several reports on somatic embryogenesis and plant regeneration in different citrus species and cultivars [6-14].

Low frequency embryo development, absence of synchrony and occurrence of embryo abnormalities are common in somatic embryogenesis of citrus [14-16]. Commercial application of somatic embryogenesis in citrus is restricted due to poor and non uniform quality of the embryos produced. Frequency of somatic embryos produce is dependent on culture conditions in which the callus is cultivated along with genetic and physiological factors [14]. Somatic embryos must develop fully and attain physiological maturity like zygotic embryos before germination [17]. Development and maturation of somatic embryos are dependent on specific physical stresses [18]. These stresses may minimize the rate of abnormal somatic embryo development. Stress treatments have been successfully used to increase the induction rates, maturation and germination of somatic embryos [19-20]. Among different stress treatments desiccation was reported to be beneficial for embryogenesis and plant regeneration in cassava [21], chestnut [22], rice [18], sugarcane [23] and wheat [24]. Chilling treatment affects several cellular processes during somatic embryo development such as cell division, differentiation, cell shape determination and cell wall deposition [25]. Low temperature treatment results in to modification of cell wall and plasma membrane interactions which in turn, leads to changes in gene expression and synthesis of products that are responsible for embryogenesis [26]. It promotes up-regulation of genes, which encodes for stress-induced proteins which are necessary for successful embryogenesis [20, 27-29].

We have previously reported the effect of storage conditions on germination of encapsulated and non-encapsulated somatic embryos [5]. In the present study, the effect of desiccation

and chilling treatments on somatic embryo development and germination was reported with an objective to test treatments that might improve somatic embryogenesis in style-derived callus cultures of rough lemon.

2. MATERIALS AND METHODS

2.1 Plant Material

Healthy rough lemon plant growing at Govt. Nursery, Department of Horticulture (Punjab), Attari, Amritsar, India was selected and used as a source of explant for raising *in vitro* cultures in February 2009. Unopened flower buds were collected and washed with teepol for 10 minutes followed by running tap water for 30 minutes. These buds were surface sterilized with 0.1% mercuric chloride for 10 min and rinsed with autoclaved double distilled water 3–4 times in laminar flow hood. Styles were excised from flower buds with a scalpel, and placed horizontally with the cut surface in contact with the medium. All media chemicals, reagents and hormones used in the present study were of analytical grade and obtained from Hi-Media, India.

2.2 Callus Induction and Proliferation

Explants were cultured on MS medium [30] containing 3% (w/v) sucrose and 0.8% (w/v) agar. Seven different media were used on the basis of supplements and growth regulators for callus induction [MS I (ME 500 $mg.L^{-1}$); MS II (500 $mg.L^{-1}$ ME and 4.52 µM 2,4-D); MS III (500 $mg.L^{-1}$ ME and 9.04 µM 2,4-D); MS IV (500 $mg.L^{-1}$ ME and 4.64 µM KN); MS V (500 $mg.L^{-1}$ ME and 9.29 µM KN); MS VI (500 $mg.L^{-1}$ ME and 8.87µM BAP) and MS VII (500 $mg.L^{-1}$ ME and 13.3 µM BAP)].The pH of the media was adjusted to 5.6 with 1 N NaOH and autoclaved at 121ºC and 15 lb in^{-2} pressure for 20 min. Single style was inoculated per tube (25 × 150 mm) containing 25 ml of the medium. For each set of experiment, 48 tubes were inoculated per replicate and experiment was repeated thrice. All cultures were maintained at 26±1ºC with a luminous intensity of 40 µ mole m^{-2} s^{-1} and 16-h photoperiod. For each culture media, number of cultures showing callus induction was recorded 30 days after initial culturing and their percent frequency was calculated.

2.3 Desiccation and Chilling Treatments

Desiccation was carried out by transferring 500 mg of embryogenic callus from culture medium to sterile empty petri dishes (100 × 15 mm) containing two sterile whatman filter papers. The petri dishes were sealed with parafilm and kept at 26±1ºC in the dark for 24, 48, 72 and 96 hours for desiccation. For chilling treatments, embryogenic callus was transferred to culture media containing double amount of agar (1.6 %) and were kept at 4ºC for 24, 48 and 72 hours. Untreated callus cultured on fresh medium served as control.

2.4 Somatic Embryo Development and Germination

Desiccated, chilled and untreated callus was transferred to fresh medium for somatic embryogenesis. The somatic embryos were allowed to mature on the same medium and the effect of desiccation and chilling treatment on somatic embryo development was recorded. For each treatment, number of cultures showing somatic embryogenesis was recorded after 3 months of culture and their percent frequency was calculated. Individual somatic embryos at advanced cotyledonary stage were picked and germination was attempted in test tubes

containing 25 ml of MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and 500 mg.L^{-1} malt extract. In another experiment somatic embryo developed from callus without treatment was cultured on same media for germination.

2.5 Moisture Content

Changes in moisture content of somatic embryos at globular, heart and torpedo stages of development were determined for each treatment by weighing before and after oven drying at 80ºC for 48 h.

2.6 Hardening and Acclimatization

The well-developed plantlets obtained from germinated somatic embryos were washed with water in order to remove adhering agar and transferred to autoclaved plastic pots containing a mixture of garden soil, sand and vermiculite in the ratio of 3:1:1. Hardening of potted plantlets was accomplished in a culture room set at 26±2ºC, 16-h day-length (40 µmole m^{-2} s^{-1}) and covered with polyethylene bags to maintain high humidity. After 12-15 days, polyethylene bags were removed initially for a short duration (15-30 min) daily for about one week. Gradually, the daily exposure time was increased by 30 min for each day. Polyethylene bags were removed after 20 days; subsequently the plantlets were transferred to the earthen pots containing only garden soil and kept for one month, and thereafter transferred to the field conditions.

2.7 Statistical Analysis

For each treatment, 24 culture tubes were inoculated and the experiments were repeated thrice. The data pertaining to callus induction, somatic embryogenesis and effect of desiccation and chilling treatment on somatic embryo development and germination were subjected to one way analysis of variance (ANOVA) and the differences among means were compared by high-range statistical domain (HSD) using Tukey's test.

3. RESULTS

3.1 Callus Induction and Somatic Embryogenesis

Callus induction was observed from styles after 2 weeks of culture on MS medium. Styles inoculated on seven media responded differently for cell proliferation. Among seven media (MS I – MS VII) tested (Table 1), maximum callus induction was observed on MS IV (81.94%) and minimum on MS III (31.24%). Compact green callus was observed when styles were cultured on 2,4-D supplemented media (MS II and III). Callus was friable and creamish-white on MS IV-VII media which was found to be embryogenic (Fig. 1A). Proembryogenic masses were developed from callus after one month of culture on fresh medium. These masses showed all stages of embryogenesis, viz., globular, heart, torpedo and cotyledonary-shaped structures. Callus produced and sub-cultured on MS II and III media generated very low frequency of proembryogenic masses which showed poor development. The effect of seven different media on somatic embryogenesis after 3 months of culture is shown in Table 1. There were significant differences in somatic embryo formation among callus formed on different media. MS II and III media showed very low

Table 1. Embryogenic callus induction and somatic embryogenesis from styles of *Citrus jambhiri* Lush

Culture Medium	[a]Callusing frequency (%)	Callus Appearance	[b]Somatic Embryogenesis (%)
MS I	54.85 ± 1.8 [c]	Friable, white	11.10 ± 1.8[d]
MS II	43.03 ± 0.7 [d]	Compact, green	6.94 ± 0.7 [d]
MS III	31.24 ± 1.2 [e]	Compact, green	4.85 ± 1.8 [d]
MS IV	81.94 ± 1.8[a]	Friable, creamy-white	18.74 ± 1.2[c]
MS V	79.16 ± 2.4 [a]	Friable, creamy-white	28.47 ± 1.8 [b]
MS VI	74.30 ± 0.7[b]	Friable, creamy-white	47.21 ±0.7 [a]
MS VII	78.46 ± 0.7 [a]	Friable, creamy white	52.08 ± 1.2 [a]
	$F_{(df\ 6,14)}$ = 185.5*; HSD = 6.89		$F_{(df\ 4,10)}$ = 183.4*; HSD = 6.55

Data shown are Mean ± SE of three experiments. Each experiment consisted of 48 replicates.
**Significant at p ≤ 0.05, [a]Observations recorded after 4 weeks of inoculation, [b]Observations recorded after 3 months of culture.*
Values followed by the same letter with in a column are not significantly different using HSD multiple comparison test.

Table 2. Effect of desiccation and chilling treatments on somatic embryo development and germination

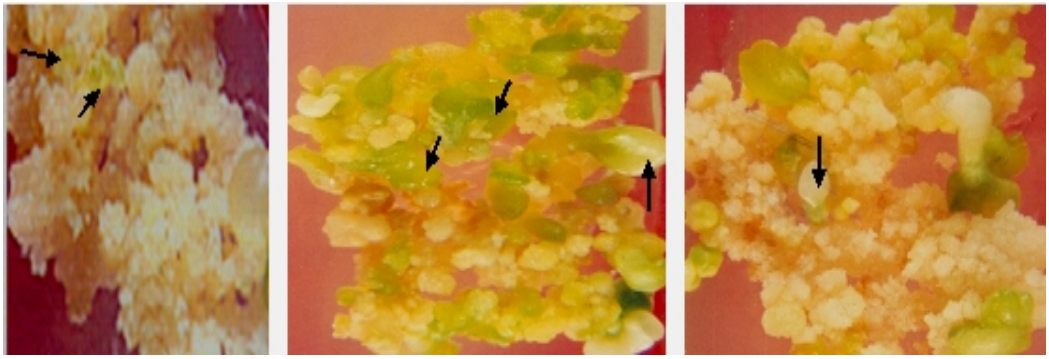
Treatment	Duration (hrs)	Culture medium	Somatic embryogenesis (%)	#Cotyledonary embryos/culture	#Abnormal embryos/culture
Untreated	-	MS VII	51.98 ± 1.4[a]	2.26 ± 0.4[c]	7.20 ± 0.4[a]
Desiccated	24	MS VII	58.33 ± 2.4[a]	6.13 ± 1.3[a]	2.80 ± 0.3[c]
	48		56.94 ± 1.4[a]	6.80 ± 1.0[a]	0.60 ± 0.3[d]
	72		47.91 ± 1.2[b]	6.60 ± 1.4[a]	0.53 ± 0.3[d]
	96		30.55 ± 1.8[c]	3.13 ± 0.4[b]	0.53 ± 0.4[d]
Chilled (4°C)	24	MS VII	49.98 ±1.8[a]	3.06 ±0.7[b]	3.40 ± 0.3[b]
	48		45.83 ± 1.2[b]	4.60 ± 1.2[b]	2.93 ± 0.4[b]
	72		52.77 ± 2.8[a]	5.33 ± 1.0[a]	2.86 ± 0.4[b]
	96		40.27 ± 1.4[b]	5.20 ± 1.3[a]	2.73 ± 0.3[c]
			$F_{(df\ 8,18)}$ = 18.7*	$F_{(df\ 8,126)}$ = 11.96*	$F_{(df\ 8,126)}$ = 181.26*
			HSD = 9.04	HSD = 1.92	HSD = 0.62

Data shown are Mean ± SE of three experiments. Each experiment consisted of 24 replicates.
**Significant at p ≤ 0.05, Observations recorded after 3 months of culture. # Average number of randomly selected 15 cultures/treatment*
Values followed by the same letter with in a column are not significantly different using HSD multiple comparison test.

frequency of somatic embryo development. Medium supplemented with 500 mg.L^{-1} ME and 13.3 µM BAP (MS VII) was effective for somatic embryogenesis among different media tested. Although this media produced low frequency of callus as compared to MS IV media, but it was more responsive towards somatic embryogenesis. Among all the media tested for callus induction and somatic embryogenesis, MS VII is selected to study the effect of desiccation and chilling on somatic embryo development.

3.2 Effect of Desiccation and Chilling on Somatic Embryogenesis

After 30 days of culture, embryogenic callus generated on MS VII media were taken out and given different desiccation and chilling treatment to study their effect on somatic embryogenesis. Table 2 shows the effect of different treatments on somatic embryo development and germination. Among desiccation and chilling treatment, former was more effective for improvement of somatic embryo development and germination in rough lemon. Embryogenic callus desiccated for 24 and 48 hours in sterile petriplates showed 58.33 and 56.94 % somatic embryogenesis respectively as compared to undesiccated callus (51.98%). After 3 months of culture there was more number of cotyledonary embryos in embryogenic cultures from desiccated callus (48 hrs) as compared to untreated callus (Fig. 1B). Increase in duration of desiccation from 24 to 96 hrs decreases the regeneration potential of embryogenic callus but there was not much significant effect on somatic embryo germination. There was significant change in number of abnormal embryos in desiccated callus ($p \leq 0.05$). Average number of abnormal embryos in desiccated callus for 48 hours was 0.60/culture as compared to 7.20/culture in untreated callus. Chilling treatment was not much effective in improvement of somatic embryogenesis but it improves the average number of cotyledonary embryos (Fig. 1C) and enhances germination frequency. Among all the treatments, desiccation for 48 hrs was proved to be effective for improvement of embryogenic potential of somatic embryos from styles of rough lemon.



A **B** **C**

Fig. 1. Somatic embryogenesis from embryogenic callus of rough lemon (Cotyledonary embryos marked) (A) Untreated callus (B) Desiccated callus (48 hours) (C) Chilled callus (72 hours)

3.3 Moisture Content of Developing Somatic Embryos

Moisture content of somatic embryos (globular, heart and torpedo stages) developed from desiccated and chilled embryogenic callus were studied (Fig. 2A & B) along with untreated callus. Moisture content was observed to be 92, 85 and 84 % respectively in globular, heart

and torpedo stages of somatic embryos developed from untreated callus cultures (control). Desiccation and chilling treatment decreases the moisture content of somatic embryos. Increase in duration of desiccation treatment decreases the moisture content of globular and heart stage somatic embryos and increase the moisture content of the torpedo stage. Somatic embryos developed from desiccated callus have less moisture content as compared to those developed from chilled callus. No significant change in the moisture content of torpedo stage of embryos developed from chilled callus was observed.

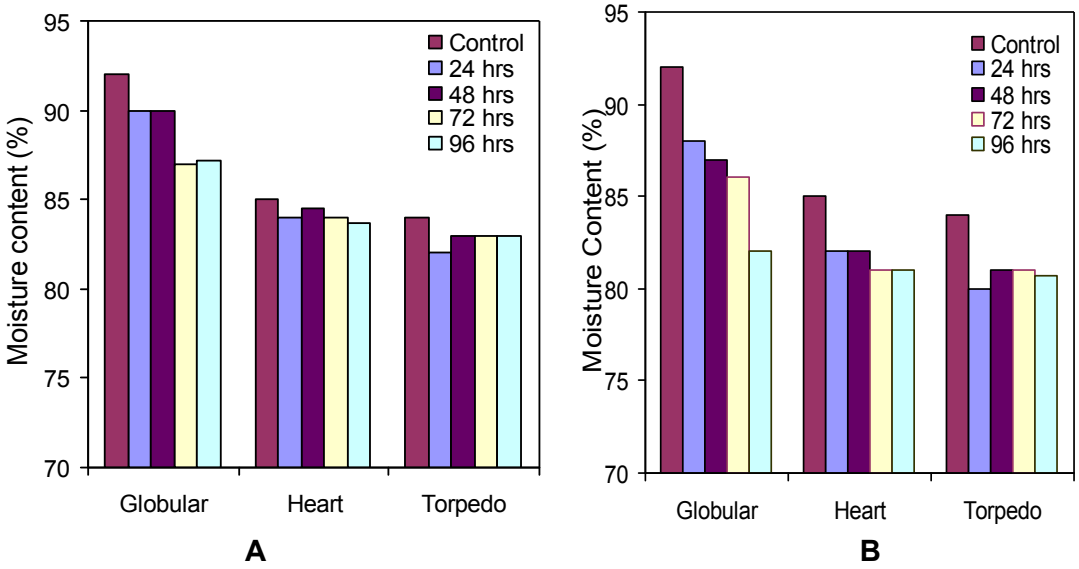
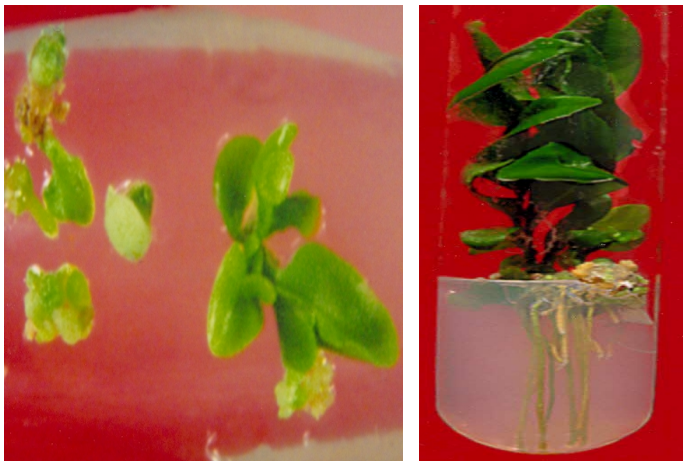


Fig. 2. Effect of desiccation (a) and chilling (b) treatment on moisture content of somatic embryos at different stages of development (Values represents mean of three observations)

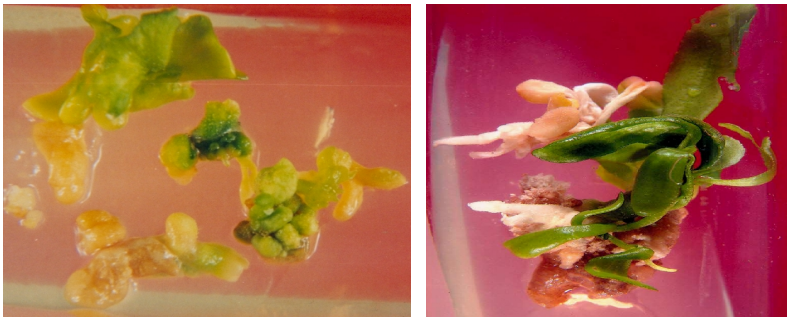
3.4 Germination of Somatic Embryos

All the somatic embryos at the cotyledonary stage were shifted to MS medium supplemented with ME (500 $mg.L^{-1}$) for germination. The somatic embryos developed from untreated, desiccated and chilled callus when germinated showed significant difference in germination. Embryos developed from desiccated callus are found to be more responsive towards germination as compared to untreated callus. Maximum (82%) somatic embryos developed from desiccated callus (48 hrs) and minimum (50%) from untreated callus germinated. Chilling treatment (72 hrs) was also effective with 71% germination frequency. Among all the treatments desiccation of embryogenic callus for 48 hrs proved beneficial for somatic embryo development and germination (Figs. 3A & B). Developmental abnormalities like pluricotyly, multiple shoot meristems, fused embryos was frequently observed in cultures regenerated from untreated embryogenic callus (Fig. 4 A). Such embryos showed abnormal germination when transferred to fresh medium (Fig. 4 B). The plantlets, thus developed, were transferred to pots containing a mixture of garden soil, sand and vermiculite (3:1:1) for acclimatization, which showed 68% survival rate. After hardening the plants were shifted to field conditions.



A B

Fig. 3. Development (A) and germination (B) of somatic embryos from desiccated (48 hrs) callus



A B

Fig. 4. Abnormal development (A) and germination (B) of somatic embryos

4. DISCUSSION

Somatic embryogenesis in genus *Citrus* was reported by many researchers in last four decades but conditions that would ensure mass regeneration of appropriate somatic embryos have not been worked out. Abnormal development and germination of somatic embryos have already been reported in citrus [31]. Some embryos produced normal shoots but poorly developed roots. In some embryos relative level of shoot and root development was not equal. Several hundred embryos were produced by embryogenic cultures during somatic embryogenesis, but fewer plants were obtained. The present investigation has shown that it is possible to improve somatic embryo development and germination in citrus from style-derived callus cultures. In previous study somatic embryogenesis from styles has been successfully used for virus elimination in citrus [7-8]. Carimi et al. [6] induced somatic embryogenesis in sweet orange navel group (*C. sinensis*) from cultures of stigma/style explants and undeveloped ovules. They reported that high frequency of style derived callus turned brown and necrotic after some time and never developed somatic embryos. They

also reported that regeneration of somatic embryos from stigma/style derived callus was lower than undeveloped ovules.

In present work, the best result for somatic embryogenesis from styles of rough lemon was obtained on MS VII media. Similar results were reported by Carimi et al [6] with the work on somatic embryogenesis from stigma/style derived cultures. Cytokinins (KN and BAP) were found to be effective in combination with malt extract (500 $mg.L^{-1}$) for somatic embryogenesis. The highest rate of callus proliferation from styles was obtained with malt extract 500 $mg.L^{-1}$ and 4.64 μM KN, but frequency of somatic embryogenesis was maximum while using MS medium supplemented with malt extract 500 $mg.L^{-1}$ and 13.3 μM BAP. Callus induced under the influence of 2,4-D (MS II and III) when transferred to fresh medium resulted in to proliferation only and very few pro-embryoids were formed. Earlier Gill et al. had also observed that 2,4-D induces non-embryogenic callus [32]. In embryogenic cell lines percentage of cultures showing somatic embryogenesis was very low and large number of embryos showed abnormal growth. Frequent abnormal somatic embryos such as fused cotyledons and altered number of cotyledons were observed and the plantlets derived from these abnormal somatic embryos showed poor germination. Only 50% of the cotyledonary embryos successfully germinated to form plantlets. The major cause for poor germination of these embryos is their failure to complete normal stages of embryogeny that are generally common to zygotic embryos. However, some studies have demonstrated that malformation does not always inhibit normal regeneration of somatic embryos [33]. Results of the present study are in conformity with previous report that cell lines derived from style-derived callus are less responsive towards somatic embryogenesis [6].

Manipulation of factors that contribute to the culture conditions was found to be effective for determination of effective conditions for somatic embryogenesis in different plant species [34]. In present study desiccation improves somatic embryo development and plant regeneration efficiency form rough lemon style-derived callus cultures. Reduced availability of water during desiccation is the most important factor in improving embryogenic response of callus cultures in terms of numbers of somatic embryos and their germinability [35]. Desiccation of callus triggers the changes in cell lines which led to differences in the soluble protein pattern [36]. It brings a shift in the developmental program of the culture from proliferation to production and maturation of embryos [37]. It was observed that desiccation treatment stimulates the normal development and maturation of somatic embryos. There was significantly less number of abnormal embryos in desiccated cell lines which was essential for high frequency regeneration of viable plantlets. There was significant change in moisture content of developing embryos after desiccation and chilling treatment. Globular and torpedo stages of somatic embryos developed from desiccated and chilled callus showed less moisture content. Low moisture content stimulates accumulation of carbohydrates, lipids and proteins in cell cultures. In earlier reports also authors reported that reduced availability of water from the medium to the callus cultures plays an important role in normal development and maturation of somatic embryos [37].

Desiccation of embryogenic callus for 48 and 78 hours was found to be effective for somatic embryo development and germination. Desiccation has been found to promote somatic embryogenesis in several plant species [18, 21-23]. The current results confirmed earlier observations that desiccation promotes somatic embryo development and maturation from embryogenic cell lines [38]. The desiccation treatments, during cell proliferation were observed to be more effective than chilling treatments. It was also noted that regeneration of somatic embryos into plantlets was significantly high in desiccated and chilled callus as compared to untreated callus. In this study chilling treatment proved to be helpful for the maturation of somatic embryos and their regeneration into plantlets. It was found to be

beneficial for embryogenesis and plant regeneration in earlier reports on Camellia [28], Pinus [25], Rape [20] and Astragalus [29]. Komamine et al. proposed that cold treatment modifies a cell wall and plasma membrane interaction which in turn, leads to changes in gene expression and synthesis of proteins that are responsible for somatic embryo formation [26]. These proteins might be playing a key role in maturation and germination of somatic embryos. Pan et al. have recently identified 24 differentially expressed proteins during the process of somatic embryogenesis in *Citrus sinensis* Osbeck [13].

5. CONCLUSION

In view of the above, it can be concluded that application of stress treatments like desiccation and chilling can be used to improve development and germination of somatic embryos in citrus from embryogenic callus. However, further investigation is required in order to understand the actual mechanism of action and factors responsible for this pathway of regeneration.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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