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Sorbitol and Sucrose- Induced Osmotic Stress on Growth of Wheat Callus and Plantlet Regeneration

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Abstract

Scutellar somatic embryos are most commonly used as a target tissue for transformation and regeneration of transgenic wheat plants. Tissue culture responsiveness of elite varieties of wheat is one of the critical factors limiting high-frequency transformation. This study investigated the influence of sorbitol and sucrose-induced osmotic stress on growth and plantlet regeneration in callus cultures of wheat *(Triticum aestivum L.* em Thell). Immature embryos, at early-medium milking stages, of field grown wheat cvs., HUW206, HUW234, Sonalika and HD2009, were induced to form the callus on MS medium supplemented with 2, 4-D and 3% sucrose alone/or in a combination of sucrose and sorbitol. Fresh weight of callus was significantly promoted when 1.5% (w/v) sorbitol was added to the MS medium in conjunction with 3% (w/v) sucrose. However, higher concentration of sorbitol concentration (3% w/v) in conjunction with 3% (w/v) sucrose did not further improve fresh weight. Sorbitol in combination with sucrose caused a decrease in the water content from calli and promoted embryogenic callus formation. After 4 weeks, a high frequency (10-25%) shoot formation was obtained on MS media, IAA 1 mgL^{-1} and zeatin 1 mgL^{-1} in supplementation with sucrose and sorbitol. However, optimum plantlet regeneration (45-65%) was found on subsequent subculture on regeneration media containing 3% (w/v) sucrose and 3% (w/v) sorbitol. Osmotic stress induced by the combination of sucrose and sorbitol is beneficial for embryogenecity of the friable calli and high-frequency plantlet regeneration.

Key words : *In vitro* selection, NaCl tolerance, Wheat, *Triticum aestivum* L.

Introduction

Wheat is the major staple food crops of the world occupying 17% of the world's cultivatable land (over 200 million hectares) (1). Due to climate change and adverse environmental conditions, conventional breeding is facing a genetic bottleneck in achieving goals of sustainable agriculture. Also, there is evidence of yield plateaus or abrupt decreases in the rate of yield gain (2). In India, wheat production in the year 2014-15 estimated at 90.78 million tonnes which is lower by 5.07 MT as compared to 2013-14. Biotechnology can be integrated with the conventional breeding to build resilient cultivars to mitigate climate change and adverse environment.

The development of efficient and reproducible *in vitro* tissue culture and plant regeneration protocols is a prerequisite for successful application of genetic transformation and regeneration of transgenic wheat. Embryogenic calli derived from scutellum are the most commonly used target tissue for wheat genetic transformation with particle bombardment or gene transfer by Agrobacterium (3-5). Although various explants sources, such as anthers (6), immature inflorescences and immature embryos (7-9), mature embryos (10-

14), immature leaves (15), mesocotyls, and apical meristems (16) have been used for callus culture in wheat. The highest frequencies of callus induction and plantlet regeneration have been obtained from the culture of immature embryos of wheat (8, 9). Despite several attempts to replace immature embryos with other explants sources, regeneration capacity from alternative explants has been low (17, 18). Till date, immature zygotic embryos remained best explant sources for embryogenic callus and plantlet regeneration in wheat. Many factors, such as developmental stage of immature embryo, culture media, genotype physiological status of the donor plants (19), the culture medium, plant growth regulators and stresses play important during somatic embryogenesis (20).

High-frequency embryogenic callus formation and embryoids development were obtained by doubling the concentration of MS salts (8), the addition of different carbohydrate sources, such as sucrose, maltose, mannitol and sorbitol and osmotic stress (21-26). Osmotic stress affects plant cells growth and physiological metabolism. However, the mechanism of osmotic stress inducing shoot regeneration has not been well investigated in wheat. Keeping in view of suggested role of osmotic stress on somatic embryogenesis, it was envisaged to investigate the effect of high osmoticum on plant regeneration in wheat. In the present study, experiments were conducted on optimization of plantlet regeneration in wheat tissue culture using the combination of sucrose and sorbitol.

Materials and Methods

***Plant Materials*:** Four elite Indian varieties of wheat cv. HUW 206, HUW 234, Sonalika and HD 2009 were used for *in vitro* tissue culture experiments. Immature embryos of field grown plants were used as starting material for *in vitro* culture.

***Callus Induction*:** Green caryopses were removed from ear heads followed by surface sterilization using 70 % ethanol, and saturated solution of sodium hypochlorite (7% w/v) for 30 s. These were then washed with several changes of sterile distilled water. Following surface sterilization, translucent immature embryos, approximately 1.5 mm in length and 1.3 mm long, were aseptically excised from green caryopses under a stereo-microscope in a laminar flow at early-medium milking stages. Subsequently, they were transferred to sterilized MS media (Murashige and Skoog (1962) (27) supplemented with 3% (w/v) sucrose and 2, 4-D 2 mgL^{-1} and solidified with 0.8 % (w/v) for callus induction. Immature embryos were cultured in Petri-dishes (90 x 15 mm) with the scutellum up and the embryo axis kept in contact with induction medium. The cultures were kept in a cooled incubator at 25°C ± 2°C. After three weeks of culture, the frequency of callus induction (CI), embryogenic callus formation (EC) and precocious germination of immature (PGIE) and somatic embryos (PGSE) were recorded.

***Histological study*:** The somatic embryogenesis in wheat was studied by histological observations of embryogenic calli sampled at regular intervals. The specimens were fixed in a formalin-acetic acid-alcohol (FAA) solution (5% formol, 5% acetic acid, 90 % of 70% ethyl alcohol; *v/v*) for at least 24 h at 4°C. Then, they were dehydrated through a graded series of ethanol and finally embedded in paraffin wax. The sections of 10 μm were cut using a rotatory microtome. Following this, they were mounted on glass slides and stained for histological analysis following the protocol of Rao (1990) (28).

***Culture Maintenance*:** After three weeks of initial callus induction, compact and nodular callus-clumps were divided into 3-4 mm sized pieces and transferred to different MS supplemented media: (a) MS media supplemented with 3% sucrose; (b) MS media supplemented with 3% sucrose+1.5% sorbitol and 2,4-D 2 mgL-1, and (c) MS media supplemented with 3% sucrose+3% sorbitol. The Frequency of friable embryogenic callus formation was scored on the basis of friable embryogenic callus formed per total number of embryogenic callus –clumps placed in culture. After three weeks of culture,

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data were recorded for fresh weight and dry weight, and the percentage water content of callus was determined as:

$$\text{Percent water content of callus} = \frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}}$$

Dry weight was determined after drying the sample to a constant weight at 80 °C.

***Plantlet regeneration*:** For regeneration, calli were transferred to MS medium with respective carbohydrate sources (sucrose 3%, sucrose 3%+sorbitol 1.5% and sucrose 3%,+3% sorbitol) supplemented with IAA 1 mgL^{-1} and zeatin 1 mgL^{-1} in a culture room maintained at 25^0C under 16/8h light/dark photoperiod. Illumination was provided by white fluorescent tubes at a photon density 70 µmol photons $m^{-2}s^{-1}$. After four weeks, plantlet regeneration frequency was calculated as the percentage of calli forming shoots. In another set of experiment, calli growing on respective carbohydrate sources were transferred to MS medium with 3% sucrose with sorbitol (1.5% and 3%, each separate treatment) supplemented with IAA 1 mgL^{-1} and zeatin 1 mgL^{-1}, and 0.8% agar in a culture room maintained in above conditions. The plantlets with well-formed roots were transferred to a sterile mixture of vermiculite containing soil (3:2) for hardening before transfer to soil.

Statistical Analysis: Data were analyzed in 2 x n Chi-square (x^2), and The individual comparisons were analyzed in 2×2 contingency table at 5% level of significance.

Results and Discussion

In the study, immature embryos (early-medium milking stages, size ~ 1.5 mm in length) readily formed the callus on MS medium supplemented with 2, 4-D 2 mgL^{-1}. Immature zygotic embryos, as well as somatic embryos have shown a tendency to germinate precociously during *in vitro* culture. Precocious germination is the development process of the somatic embryo before its complete maturation has taken place (29). In the preliminary experiment, it has been observed that precocious germination of embryos (zygotic and somatic) is influenced by genotypes. Precocious germination is an undesirable trait in tissue culture, because its expression limits the embryogenic potential of the culture. Once precocious germination is initiated, somatic embryos cannot multiply to give rise to secondary embryogenic tissues (30). After three weeks of culture, microscopic observation of the callus revealed the presence of a mixture of two types of callus: a compact, nodular embryogenic callus and a friable, watery and translucent callus (Fig. 1). In this work, all the wheat cultivars showed high-frequency callus induction (CI), ranging from 70 to 82 percent, which was independent of genotype in a Chi-square test ($p< 0.05$) (Table 1). However, the proportion of embryogenic callus formation (EC) was significantly different in wheat cultivars (d.f. 4, Chi-square test ($p< 0.05$) (Table 1). The significant influence of the genotype on embryogenic callus formation has also been observed in other studies (21, 31, 32).

Hormones play an important role in zygotic embryo and somatic embryogenesis. ABA is known for prevention of precocious germination. Approximately 90% of endogenous ABA in excised immature soyabean embryos diffused into ABA-free solution within 2 days (33). An abundant embryo protein, early methionine-labelled (Em) and wheat germ agglutinin (WGA) accumulate during mid to late stages of embryogenesis at a time when endogenous ABA levels are high in the kernel. These proteins disappear when the embryos are cultured in the absence of ABA and the same time embryos germinate prematurely (34). Therefore, it is concluded that precocious germination of cultured immature embryos may be related to loss of endogenous ABA through diffusion into ABA free medium. When immature embryos at 14 days post anthesis (DPA) were dissected from immature grains and cultured on nutrient medium, they commenced precocious germination immediately. This germination was

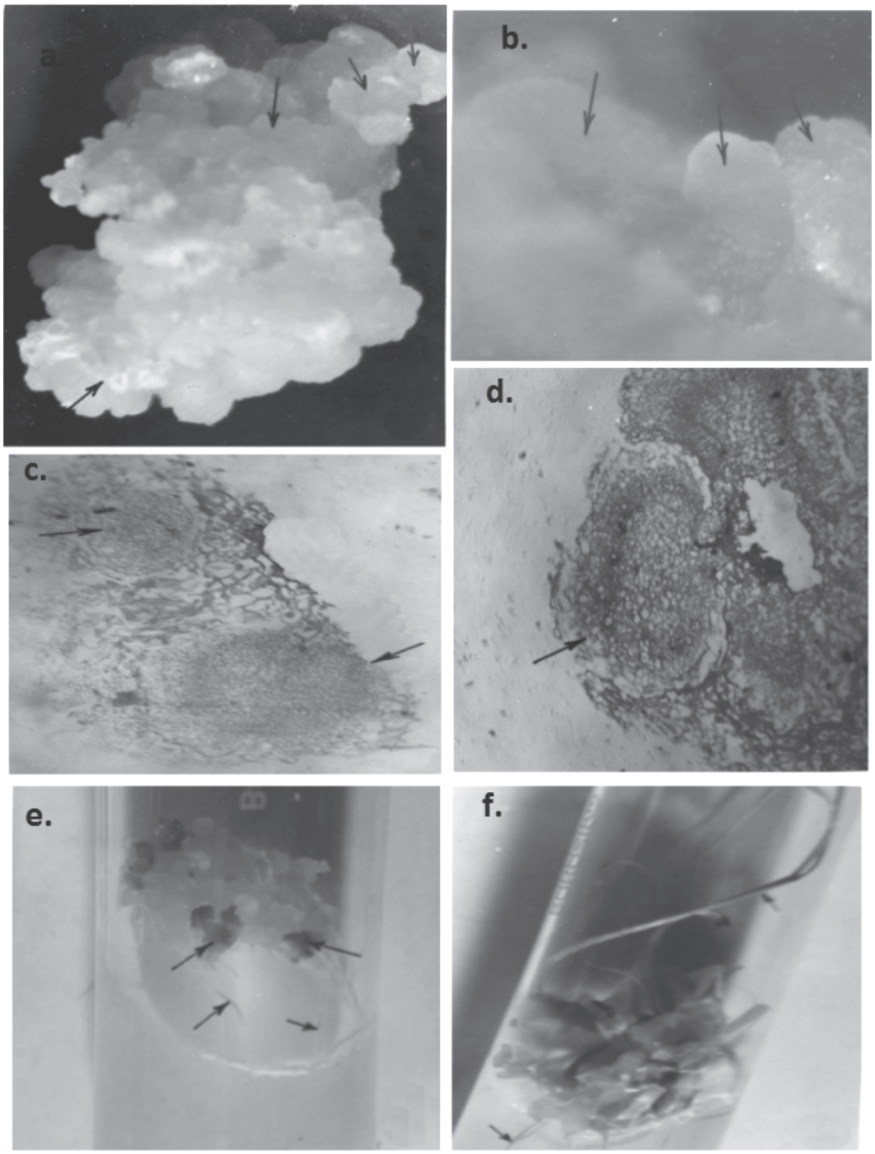

Fig. 1. Embryogenic callus of wheat at different developmental stages of embryoids and plantlet regeneration (a. embryogenic callus; b. A portion of calli showing somatic embryos at globular stage; c. Section of embryo at the globular stage; d. Post globular development; e. Embryogenic calli showing green spots (at early regeneration stage); and f. Plantlet regeneration.

prevented, not only by the inclusion of abscisic acid in the medium but also by the inclusion of osmotically active agents (35). There are reports indicating that osmoticum and/or exogenous ABA can prevent precocious germination, and enhance embryogenic callus formation (9,21,34,36-37).

Effect of Sugar and Sorbitol Induced Osmotic stress on Callus Growth: In most of the cereals, the subculture of primary embryogenic culture

Table 1. Frequency of precocious germination immature embryo (PGIE), callus induction (CI), embryogenic callus formation (EC) and precocious germination of somatic embryos on MS medium supplemented with 2, 4-D 2 mgL^{-1}

Genotypes	PGIE	CI	EC	PGSE
HUW 206	0.30 C	80 A	0.65 A	0.06 CD
HUW 234	0.56 B	75 A	0.50 B	0.12 BC
Kalyansona	0.06 D	78 A	0.70 A	0.04 D
HD 2009	0.84 A	70 A	0.25 C	0.50 A
Sonalika	0.20 C	82 A	0.75 A	0.20 B

Data were analyzed by chi -square test (χ^2) test in 2×n contingency table, and the pair- wise comparison each column were made in 2×2 contingency table. The values in the column followed by the same letter are not significantly different $p< 0.05$. PGIE=precocious germination of immature embryo; CI= callus induction; EC= yellow scutellar callus formation; PGSE= precocious germination of somatic embryos.

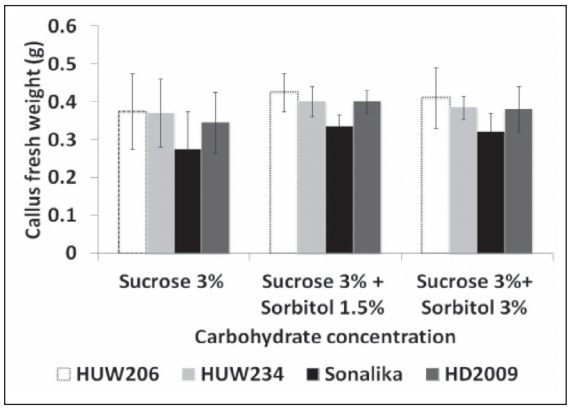

Fig. 2. Fresh weight of calli of four different cultivars of wheat on MS medium supplemented with 3% sucrose alone, and in conjunction with sorbitol supplementation (1.5% and 3%)

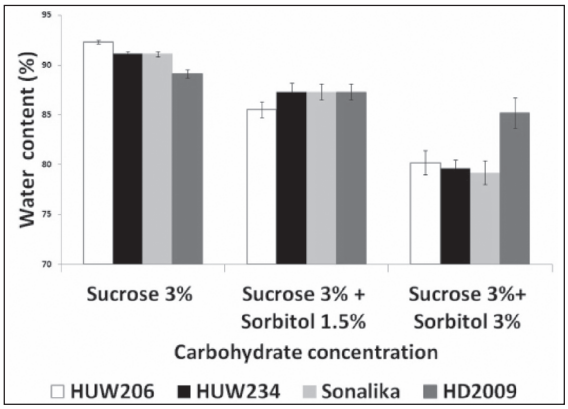

Fig. 3. Water content in calli of four different cultivars of wheat on MS medium supplemented with 3% sucrose alone, and in conjunction with sorbitol supplementation (1.5% and 3%)

leads to the formation of friable calli. A cluster of somatic embryos, obtained on callus induction medium, on subsequent subculture on combinations of sucrose and sorbitol formed friable callus. In the present work, fresh weight of friable callus was significantly promoted when 1.5% sorbitol was added to the MS medium in conjunction with 3% sucrose. With 1.5% sorbitol, the percent increase in fresh weight was highest in wheat cv. Sonalika (21.8%) followed by HD2009 (15.9%) and HUW206 (13.3%) and least in HUW234 (8.1%) as compared to fresh weight response to 3% sucrose. However, an additional increase in sorbitol to 3% in conjunction with sucrose (3%) did not further improve fresh weight. It appears that the promotory effect of sucrose on friable callus growth diminished with high concentrations of sorbitol, above 1.5% (Fig. 2). Generally, as the concentration of sorbitol increased, the water content of friable callus decreased. When 1.5% sorbitol was added in conjunction with 3% sucrose in the medium,

water content decreased ranging from 2 to 7.3 %, highest in wheat cv.HUW 206 followed by HUW 234 (4.7%), Sonalika (4.1%), and least in HD2009 as compared to water content at 3% sucrose. The Water content of wheat friable callus significantly decreased with further increase in sorbitol to 3%, ranging from 4.3% to 13.1%, highest in wheat cvs. HUW206 and Sonalika followed by HUW 234 (12.6%) and least in HD2009 (Fig.3).

Improvement in embryogenic callus formation in wheat has been demonstrated by application of various sugars, such as sucrose (38) and maltose (39), sorbitol (40), mannitol (41), and PEG (41) and as well as high MS salt concentration (8, 9). Sorbitol used in conjunction with sucrose had beneficial effects on morphogenesis of rice (42,43), wheat (40, 44), and barley cultures (40). However, there is no evidence that sorbitol is utilized as a carbon source in these species. Rather, sorbitol was to metabolized in maize and imparted beneficial effects on embryogenic callus formation(45). It is reported that water-stress induced plantlet regeneration in rice (46-48).

***Effect of Sugar and Sorbitol Induced Osmotic Stress on Plantlet Regeneration*:** Friable- calli of wheat growing on sucose-sorbitol containing media showed plantlet regeneration ranging from ranged from 17 to 25% on sugar and sorbitol supplemented MS media after four weeks of culture (Fig. 4). However, high- frequency plantlet regeneration occurred on prolonging osmotic stress of calli (eight weeks of culture) under sucrose-sorbitol induced osmotic stress (Fig.5).

Under osmotic stress, the incessant supply of endogenous ABA favoured the development and maturation somatic embryos leading to high-frequency somatic-embryo to plant conversion. It is reported that osmotic stress for an extended period to the cultured embryos of wheat has promoted expression of Em genes under continued presence of embryonic ABA (35). Another report also supported embryonic ABA synthesis in wheat under drought condition (49).

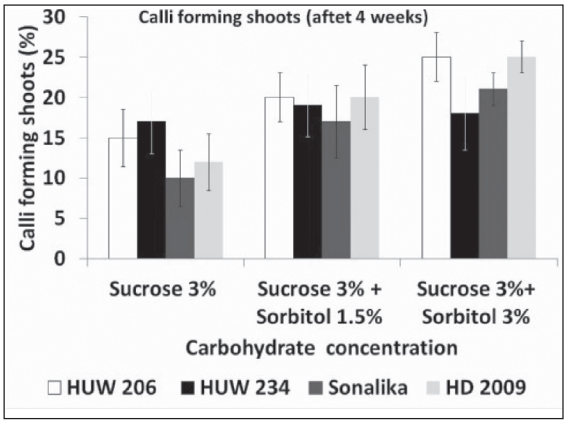

Fig. 4. Calli forming shoots after four weeks of culture on MS medium supplemented with 3% sucrose alone, and in conjunction with sorbitol supplementation (1.5% and 3%)

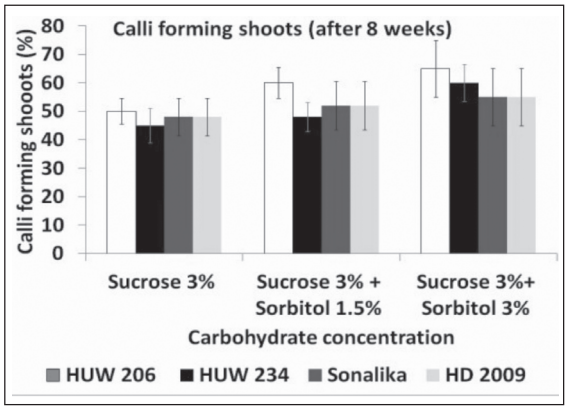

Fig. 5. Calli forming shoots after eight weeks of culture on MS medium supplemented with 3% sucrose alone, and in conjunction with sorbitol supplementation (1.5% and 3%)

Thus, sucrose and sorbitol-induced osmotic stress enhanced somatic embryogenesis and shoot regeneration in immature embryo culture of wheat.

Conclusion

ABA-mediated stimulation of somatic embryogenesis is implicated in wheat. However, the mechanism of osmotic stress induced signaling on regeneration in wheat still needs to be further explored.

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References

1. Atchison, J., Head, L. and Gates, A. (2010). Wheat as food, wheat as industrial substance; comparative geographies of transformation and mobility. Geoforum, 41: 236–46.
2. Grassini P, Eskridge, K.M. and Cassman, K.G. (2013). Distinguishing between yield advances and yield plateaus in historical crop production trends. Naure Communication, 4: 2918.
3. Shewry Jones, P.R. (2005). Transgenic wheat: Where do we stand after the first 12 years?. Annals Applied Biology, 147: 1–14.
4. Sparks, C.A. and Jones, H.D. (2009). Biolistics transformation of wheat. Methods in Molecular Biology, 478:71–92.
5. Xia, L., Ma, Y., He, Y. and Jones, H.D. (2012). GM wheat development in China: current status and challenges to commercialization. Journal of Experimental Botany, 63: 1785–90.
6. Brisibe, E.A., Gajdosova, A., Olesen, A. and Andersen, S.B. (2000). Cytodifferentiation and transformation of embryogenic callus lines derived from anther culture of wheat. Journal of Experimental Botany, 51: 187–196.
7. Ozias-Akins, P. and Vasil, I.K. (1982). Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): Evidence for somatic embryogenesis. Protoplasma, 110: 95–105.
8. Ozias-Akins, P. and Vasil, I.K..(1983). Improved efficiency and normalization of somatic embryogenesis in *Triticum aestivum* (wheat). Protoplasma, 117: 40–44.
9. Redway, F.A., Vasil, V., Lu, D. and Vasil, I.K. (1990). Identification of callus types for long-term maintenance and regeneration from commercial cultivars of wheat (*Triticum aestivum* L.). Theoretical and Applied Genetics, 79: 609-617.
10. Özgen, M., Türet, M., Altýnok, S. and Sancak, C. (1998). Efficient callus induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes. Plant Cell Reports, 18: 331–335.
11. Delporte, F., Mostade, O. and Jacquemin, J.M. (2001). Plant regeneration through callus initiation from thin mature embryo fragments of wheat. Plant Cell Tissue and Organ Culture, 67: 73–80.
12. Zale, J.M., Borchardt-Wier, H., Kidwell, K. K. and Steber, C.M. (2004). Callus induction and plant regeneration from mature embryos of a diverse set of wheat genotypes. Plant Cell Tissue and Organ Culture, 76: 277–281.
13. Chen, J. Y., Yue, R. Q., Xu, H. X. and Chen, X.J. (2006). Study on plant regeneration of wheat mature embryos under endosperm-supported culture. Agricultural Sciences in China, 5: 572–578.
14. Parmar, S. S., Sainger, M., Chaudhary, D. and Jaiwal, P.K. (2012). Plant regeneration from mature embryo of commercial Indian bread wheat (*Triticum aestivum* L.) cultivars. Physiology and Molecular Biology Plants, 18: 177–183.
15. Wang, C. T. and Wei, Z.M. (2004). Embryogenesis and regeneration of green plantlets from wheat (*Triticum aestivum*) leaf base. Plant Cell Tissue and Organ Culture 77:149–156.
16. Ahmad, A., Zhong, H. and Wang W. (2002). Shoot apical meristem: *In vitro* regeneration and morphogenesis in wheat (*Triticum aestivum* L.). *In Vitro Cellular and Developmental Biology-Plant,* 38: 163-167.

17. Dodig, D., Zoriæ, M., Mitiæ, N., Nikoliæ, R. and King, S.R. (2010). Morphogenetic responses of embryo culture of wheat related to environment culture conditions of the explant donor plant. Scientia Agricola, 67: 295–300.

18. Hafeez, I., Sadia, B., Sadaqat, N. A., Kainth, R. A., Iqbal, M. Z. and Khan, I.A. (2014). Establishment of efficient *in vitro* culture protocol for wheat land races of Pakistan. African Journal of Biotechnology, 11: 2782–2790.

19. Pastori, G.M., Wilkinson, M.D., Steele, S.H., Sparks, C.A., Jones, H.D. and Parry, M.A.. (2000). Age dependent transformation frequency in elite wheat varieties. Journal of Experimental Botany, 52: 857–863.

20. Zavattieri, M., Frederico, A., Lima, M., Sabino, R. and Arnholdt-Schmitt, B. (2010). Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron Journal of Biotechnology, 13:12-13.

21. Carman, J.G. (1988). Improved somatic embryogenesis in wheat by partial simulation of the in-ovulo oxygen, growth-regulator and desiccation environments. Planta, 175: 417-424.

22. Altpeter, F., Vasil, V. and Srivastava, V. (1996). Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Reports, 16: 12-17.

23. Patnaik, D., Mahalakshmi, A. and Khurana, P. (2005). Effect of water stress and heavy metals on induction of somatic embryogenesis in wheat leaf base cultures. Indian Journal of Experimental Biology, 43: 740–745.

24. Skrzypek, E., Szechyñska-Hebda, G. D. and Goc, A. (2008). The role of osmotic stress during in vitro regeneration of *Triticum aestivum* L. and *Vicia faba* ssp. minor. Zeszyty Problemowe Postêpów Nauk Rolniczych, 524.

25. Javed, F. and Ikram S. (2008). Effect of sucrose induced osmotic stress on callus growth and biochemical aspects of two wheat genotypes. Pakistan Journal of Botany, 40: 1487–1495.

26. Hassan, M., Ahmed, Z., Munir, M., Malik, S. I. and Shahzad, K. (2009). Effect of sorbitol in callus induction and plant regeneration in wheat. African Journal of Biotechnology, 8(23).

27. Murashige, T. and Skoog F.(1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum, 15: 473–497.

28. Rao, J.D. (1990). Tissue culture studies in different genotypes of wild and cultivated chickpea. PhD Dissertation, Osmania University, Hyderabad, India. Pp. 47–49.

29. Sharmila, A., Bapat, S.A., Joshi, C.P. and Mascarenhas, A.F. (1988). Occurrence and frequency of precocious germination of somatic embryos is a genotpye dependent phenomenon in wheat. Plant Cell Reports, 7:538-541.

30. Lange, C.E., Federizzi, L.C., Carvalho, F.I.F., Dornelles, A.L.C. and Handel, C.L. (1998). Genetics of *in vitro* organogenesis and precocious germination of wheat embryos. Genetics and Molecular Biology, 21(1).

31. Barro, F., Martin, A., Lazzeri, P.A. and Barcelo P. (1999). Medium optimization for efficient somatic embryogenesis and plant regeneration from immature inflorescences and immature scutella of elite cultivars of wheat, barley and tritordeum. Euphytica. 108: 161–167.

32. Daðüstü, N. (2008). Comparison of callus formation and plant regeneration capacity from immature embryo culture of wheat (*Triticum aestivum* L.) genotypes. Biotechnology and Biotechnological Equipment, 22: 778–781.

33. Ackerson, R.C. (1984). Abscisic acid and precocious germination in soybeans.

Journal of Experimental Botany, *35*(3):414-421.

34. Qureshi, J.A., Kartha, K.K., Abrams, S.R. and Steinhauer, L. (1989). Modulation of somatic embryogenesis in early and late-stage embryos of wheat (*Triticum aestivum* L.) under the influence of (±)-abscisic acid and its analogs. Plant Cell Tissue and Organ Culture, 18(1):55-69.

35. Morris, P.C., Kumar, A., dianna j. Bowles, D.J. and Cuming, A.C. (1990). Osmotic stress and abscisic acid induce expression of the wheat Em genes. Eur. J. Biochem., 190: 625-630.

36. Galiba, G. and Yamada, Y. (1988). A novel method for increasing the frequency of somatic embryogenesis in wheat tissue culture by NaCl and KCl supplementation. Plant Cell Reports, 7(1):55-58.

37. Brown, C., Brooks, F.J., Pearson, D. and Mathias, R.J. (1989). Control of embryogenesis and organogenesis in immature wheat embryo callus using increased medium osmolarity and abscisic Acid. Journal of Plant Physiology , 133(6):727-733.

38. Rasco Gaunt, S., Riley, A., Cannell, M., Barcelo, P. and Lazzeri, P.A. (2001). Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum* L.) varieties via particle bombardment. Journal of Experimental Botany, 52: 865–874.

39. Souza Canada, E.D. and Beck E. (2013). Embryogenic callus induction on the scutellum and regeneration of plants as basis for genetic transformation of spring wheat (*Triticum aestivum* L.) cultivars from Argentina. Journal of Basic and Applied Genetics, 24: 55–66.

40. Ryschka, S., Ryschka, U. and Schulze, J. (1991). Anatomical studies on the development of somatic embryoids in wheat and barley explants. Biochemie und Physiologie der Pflanzen, 187: 31–41.

41. Brown, C., Brooks, F. J., Pearson, D. and Mathias, R.J. (1989). Control of embryogenesis and organogenesis in immature wheat embryo callus using increased medium osmolarity and abscisic acid. Journal of plant Physiology, 133: 727–733.

42. Kishor, P.B.K. and Reddy, G.M. (1986). Retention and revival of regenerating ability by osmotic adjustment in long-term cultures of four varieties of rice. Journal of Plant Physiology, 126: 49–54.

43. Al-Khayri, J. and Al-Bahrany, A. (2002). Callus growth and proline accumulation in response to sorbitol and sucrose-induced osmotic stress in rice. Biologia Plantarum 45: 609–11.

44. ul Hassan, M., Ahmed , Z., Munir, M., Malik, S.I. and Shahzad K.(2009). Effect of sorbitol in callus induction and plant regeneration in wheat. African Journal of Biotechnology, 8: 6529–6535.

45. Swedlund, B. and Locy, R.D. (1993). Sorbitol as the primary carbon source for the growth of embryogenic callus of maize. Plant Physiology, 103: 1339–1346.

46. Lai, K.L. and Liu, L.F.(1988). Increased plant regeneration frequency in water-stressed rice tissue cultures. Japanese Journal of Crop Science 57: 553–557.

47. Tsukahara, M. and Hirosawa T.(1992). Simple dehydration treatment promotes plantlet regeneration of rice (*Oryza sativa* L.) callus. Plant Cell Reports, 11: 550–553.

48. Geng, P.P., La, H., Wang, H. and Stevens, E.J.C. (2008). Effect of sorbitol concentration on regeneration of embryogenic calli in upland rice varieties (*Oryza sativa* L.). Plant Cell Tissue and Organ Culture, 92: 303–313.

49. Singh, P., Bhaglal, P. and Bhullar, S. (2000). Wheat germ agglutinin (WGA) gene expression and ABA accumulation in the developing embryos of wheat (*Triticum aestivum*) in response to drought. Plant Growth Regulators, 30: 145-150