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Salicylic acid improves somatic embryogenesis in callus derived from mature embryos of *triticale*

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Abstract

The main aim of this study was to define impacts of salicylic acid (SA) on the somatic embryo formation ratio in *triticale*. Mature embryos, obtained from seeds, were grown on Murashige and Skoog (MS) medium containing 1 and 2mM SA + 1 mg mL-1 2,4-D. SA promoted the somatic embryo formation ratio in both concentrations. Green spots were seen on the callus tissues and developed easily in the SA media. Some physiological changes were assayed in both somatic embryos and callus tissues. The results demonstrated that H2O2 and MDA were promoted in somatic embryo tissues. In outcome, it was considered in this study that *in vitro* SA might be used to increase somatic embryo number in callus samples of *triticale*.

Keywords: Salicylic acid, *triticale*, somatic embryo

Introduction

Salicylic acid (SA) is one of the plant growth regulators that naturally belongs to the group of phenolic acids the plant. SA has many important physiological functions in a variety of plant products, where it contributes to increasing the plant's tolerance to systemic-acquired resistance (1). Many reports have been revealed a correlation between endogenous hormone levels in cells and external environments (2, 3, 4, 5). SA pretreatment not only regulated the activity of ROS-metabolism, but also changed the transfer of nutrient and basal metabolisms, such as cell division, and stomatal balance. It also acts as a positive the regulator in embryogenic callus proliferation and regeneration response of plants (6). Nonetheless, the functional relationship between SA and somatic embryo formation regulating embryogenic differentiation under tissue culture remains uncertain. Somatic embryogenesis (SE) is the development procedure in which an explant is derived from a single embryogenic differentiation capability or maternal tissues. Somatic embryogenesis frequently comprises indirectly via a replacing callus stage or directly from premature explants (7). *In vitro*, SE methods have been performed

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on many plant species. SE is a crucial step for plant regeneration and is identified as a potential model system for indicating the morphological, physiological, molecular, and biochemical situations that begin during the plant cell cycle (3, 7). It is also a beneficial attempt at regeneration in biotechnology-supported cultivar improvement (8). SE is stimulated tissue culture when the explants are subjected to exogenous hormones mostly the 2,4-D and abiotic stresses (9), such as heat stress, chemical usage, and osmotic stress. *Triticale* is grown for its human and animal feed and cold resistance, higher growth performance, and tolerance to many abiotic stresses (10). The medium combinations, concentrations and type of hormones, microelements, pH, carbohydrates, physical conditions, and other development supplements are among the determinative *in vitro* experiment factors in plant species. Modulation of combinations in culture media is basic to induce embryogenic callus dedifferentiation and most of the studies are devoted to optimizing the hormones. Some external phytohormone applications and other agents have also been reported to usually affect embryogenesis from somatic tissues. Earlier studies indicated the involvement of reactive oxygen species in somatic embryogenesis mechanisms. A number of the significant events in the somatic embryogenesis process including dedifferentiation of cells, metabolomic signaling pathways, and programmed cell death have been studied to be affected by oxidative stress (11). Although earlier studies have emphasized the double function of ROS in embryogenic callus that are both inducing and toxic, a few has been reported on the ROS impacts on *in vitro* SE procedures. The study aimed was to increase the capacity of somatic embryo formation for *triticale* by investigating different SA concentrations in the embryogenic callus medium. Moreover, it was to determine the degrees of the antioxidant system in the *triticale* samples.

Materials and Methods

Plant material and callus induction: In our report, Mature embryos of the Ümran Hanım genotype were obtained as the explant for the response to callus initiation. The seeds were disinfected with 22% NaOCl for 20 min, and washed two times with ddH2O water. They were kept in sterile distilled water at 25°C overnight in the dark and mature embryos were removed. Mature embryos were placed in sterile medium from two weeks old plants onto hormone-free MS medium (Murashige and Skoog 1962) (12) including MS basal medium (pH 5.7) and 8 g agar added with 1 mg mL-1 of 2,4-D. The explants were put in total darkness at 25 ± 1 °C controlled conditions for a month. After that, callus formation was assessed and used for embryogenic callus studies.

Salicylic acid treatment: SA were dissolved in ethanol (99 %) in the dark using gentle shaking at room temperature. The pH was adjusted to 6.0 with 1 N NaOH and 1 N HCl and maintained in a refrigerator at -20 °C until needed. For the SA treatment, callus was transferred in presence of different levels of SA (0, 1.0, and 2.0 mM) for three time periods (7, 14 and 21 days) and incubated at 26 °C in a growth chamber for a month. The callus was kept for every 3 weeks in the sub-culture medium. The experiment period was 3 months. The somatic callus was determined for somatic embryo formation at the end of 3 months.

H_2O_2: H_2O_2 (Hydrogen peroxide) content was measured using the method of Sergiev et al. (1997). 0.2 g of callus material was taken and 5 mL of cold Trichloroacetic Acid (TCA, 0.1%) was homogenized. The obtained homogenate was centrifuged at 12,000 x g for 15 minutes at 4°C. Then 0.5 mL of 10 mM KH2PO4 (pH 7.0) buffer and 1 mL of KI were added to 0.5 mL of the obtained supernatant. Absorbent values were measured and recorded at 390 nm (13).

Malondialdehyde (MDA): Malondialdehyde was analyzed using the method of Heath and Packer (1968) using liquid nitrogen. After taking 0.4 g from the *triticale* callus and homogenizing in 4 ml of 0.1% TCA, the homogenate was centrifuged at 13,000 rpm for 15 minutes. 1 ml of the supernatant part of the tube was taken and 1 ml of 0.5% TBA (Thiobarbituric Acid) solution was joined to it. The reagent mixture was kept in boiling water for 40 minutes and the reaction was stopped by placing the tubes in an ice bath. The samples were centrifuged at 12 000 rpm for 5 minutes and the supernatant was taken and the absorbance value of 532 nm and the absorbance value for non-specific absorption at 600 nm were read (14).

SEM: Callus samples were incubated in a solution of 5% glutaraldehyde (pH 7.2, 0.1 M phosphate buffer) for 2h at room conditions. Following, the dehydration step using a graded ethanol series, water was removed from samples with a CPD (CO_2 critical-point drying) system, sputter-coated with gold (Jeol JFC-1100 E ion-sputtering system) and samples were detected with a scanning electron microscope (HITACHI S-4700).

Statistical Analysis: Each analysis was repeated three replications. Analysis of variance was carry out using a one-way ANOVA test using SPSS 13 software and averages were evaluated by the Duncan test at the $P<0.05$ confidence level.

Results

Somatic embryo formation capacity: Fifty explants were cultured in 100x15 mm petri plate (sealed with Parafilm) including 30 ml of 1 and 2mM salicylic acid media and the petri plate were transferred in a growth cabinet at 28 °C and with a 16 h light/8 h control system provided by cool-white fluorescent light. On day ten, most explants were expanded, and on day 15, callus began to appear. By one month, some callus was obtained and with fluffy, globular, hard, and white appearance, defined as non-embryogenic callus (Figure 1). Supplementation of the callus formation medium with SA exhibited a remarkable increase in somatic embryos at the concentration of 2.0 mM of SA while 1 mM concentration, although increased the somatic embryos but not up to a significant level. It was detected that the formation of somatic embryos in genotype was greatly linked to exogenous growth regulator under aseptic conditions and the lowest somatic embryo formation was observed in absence of SA (Table 1).

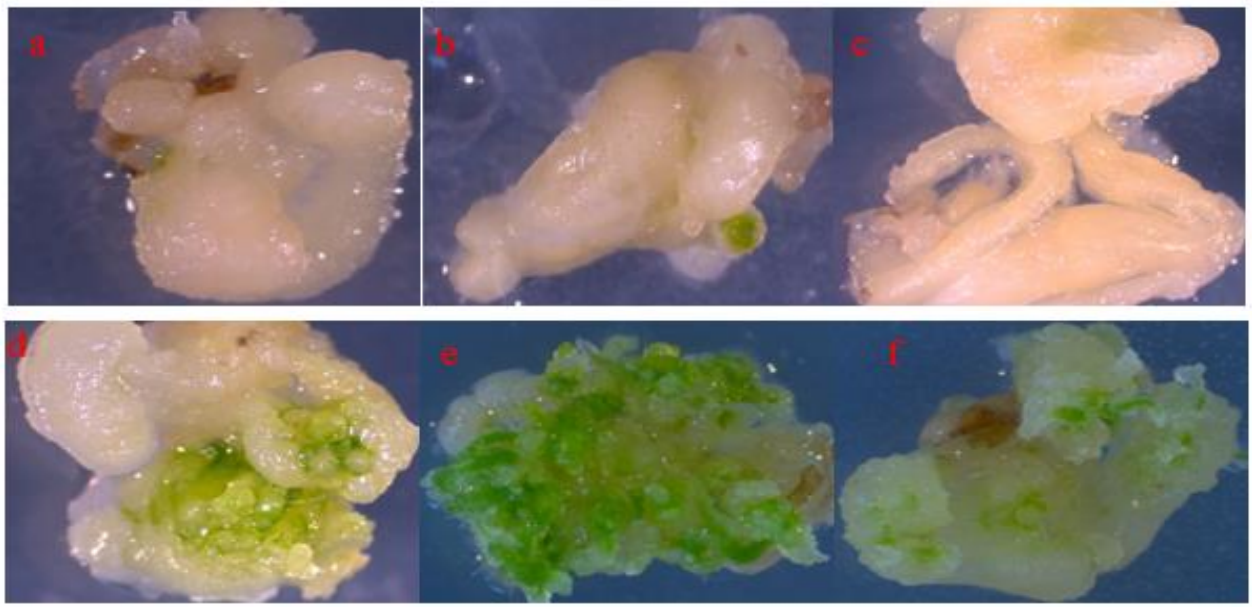


Figure 1. Callus of *triticale* species a: Control, b: 1 mM SA, c: 2 mM SA, embryo *triticale* species c: Control, d: 1 mM SA, e: 2 mM SA.

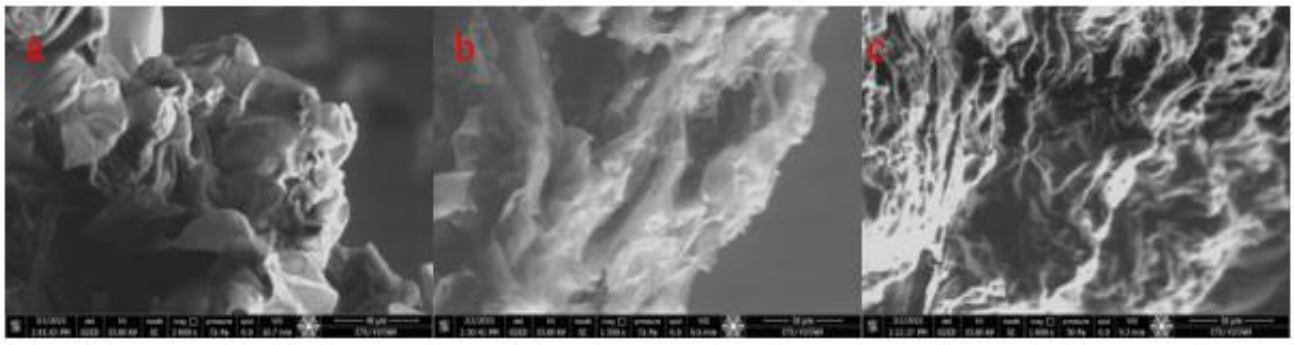
Table 1. Changes in somatic embryo formation in *triticale* genotype (Ümran Hanım) supplementation salicyclic acid

Genotype Ümran Hanım	Explant number	Embryogenic callus	Somatic embryos	H_2O_2	MDA
Control	50	19	19(7)	0.006±0,0000[b]	0.129±0.0066[c]
1mM SA	50	24	24(21)	0.026±0.0000[b]	0,2667±00088[b]
2mM SA	50	35	35(44)	0.130±0.0146[a]	0.570±0.0115[a]

[a-b-c]Means in the same column with different superscript letters differ significantly ($P<0.05$)

SEM analysis: SEM analysis indicated that embryogenic callus formation had been created in the callus tissues a cultivated on SA medium for 14 days (Figure 2), but no further improvement was observed in this phase. The results showed that the somatic embryo development appears in SA treatment callus tissues. Globular callus structures were obtained in control samples (Figure 2a). Histological tissues showed that somatic embryo formation and improvement were obtained in the callus grown on 1 mM SA and 2 mM SA media for 14 days (Figure 2b, and c). The function of SA in the embryogenesis of *triticale* was investigated and indicated that the presence of low concentrations of SA was necessary for somatic embryo formation from callus tissues, and the presence or absence of lower amounts of SA in the medium affected somatic embryos development and maturation.

Figure 2. Image of callus structures of SEM analysis (a: Control, b: 1 mM SA, c: 2 mM SA).



H_2O_2 and MDA: Cell damage was increased due to increased SA concentration in H_2O_2 and MDA. 1 mM SA showed the least damage to the cell. H_2O_2 varied between 0.006 and 0.130 mg/g FW values. 2 mM SA differed from the control. On the other hand, MDA was varied between 0.129 and 0.570 mg/g FW values. All SA concentrations differed from the control (Table 1).

Discussion

As *triticale* is a main field crop plant worldwide, several efforts have been tried to provide higher yields and quality. Nevertheless, its ploidy character and its genotypic base and other phenotypic base have placed restrictions on the ability of breeding to control its regeneration and production in the field and in changing natural growing conditions. Therefore the application of the tissue culture system can be benefical for the genetic improvement of *triticale*. The *in vitro* culture conditions can be impacted by several traits, such as plant type, initiation material, medium conditions, and growth parameters (10). The combination of auxin and auxin-like derivatives including SA, and ABA increased the callus formation ratio in many plant cultivars. SA has been shown as a real factor in callus induction and somatic embryogenesis (3). It was found that SA application promoted response to somatic embryo formation rate in two weeks in our study (Table 1). Our outcomes agree with those reported by Hao et al., (2006) (4) in the study on naked oat with the supplementation of SA at

0.5 mM *in vitro* culture medium. Their results indicate that SA promoted the ratio of somatic embryo formation compared with the control medium. However, embryogenic callus initation and plant regeneration were enhanced by 43% and 61%, and these were a 5- and 4-fold promote as compared to untreatments on medium without SA. This can be explained that somatic embryo formation is greatly linked to a synergistic action between SA and embryogenic callus formation. Our results suggest that H2O2 highly accumulated in only higher SA concentration during the short period, but more influencely saved during SA applications at 1 mM. However, changes in the intracellular SA level affected the H2O2 content in callus culture (Table 1). The accumulation of H2O2 in the short term observed in somatic embryos is likely because of the osmotic stress stimulated by the exogenous hormone treatment. MDA accumulation is externalized to be a marker of oxidative stress. In SA treatment callus, MDA was remarkably promoted under *in vitro* culture and the impacts of SA on MDA accumulation are well related to the somatic embryo formation ratio. These results are consistent with the outcomes obtained by SEM analysis. In SEM analysis, among the two different SA concentrations, a largely different callus structure was detected in their control callus (Fig 2). Activating antioxidants in cell tissues that clean ROS adversely impacts biochemical processes in tissues that are crucial for cell differentiation. Overexpression of ROS is related to *in vitro* growth and is urgent in the early phages of shoot regeneration (15). SA has been shown to inhibit the activities of APX (16), and SOD (17) and promote the activity of CAT (18), in this way, leading to endogenous H2O2 accumulation. Our outcome is consistent with this results. It is recommened that somatic embryogenesis may be related to the reactive oxygen species degree in the callus, namely higher MDA and higher H2O2 level positive for embryogenic callus formation, and a crucial act in the foundation of polarity at early phages of plant embryogenesis in *triticale*. However, from the results provided here, it is not certain whether reactive oxygen species are the reason or the outcome of the embryogenic callus phage, and further researches have to be performed.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

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