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Full Length Article

The Effect of Exogenous Proline and Salicylic Acid Application on Proline and Apoplastic Protein in Cold Tolerance of Pepper Callus Cultures

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Abstract

In this study, the effect of proline and salicylic acid treatment on the amount of proline and apoplastic protein in cold tolerance of pepper callus cultures was investigated. Callus tissue developing in magenta boxes was transferred to an MS medium containing SA (0.25 and 0.50 mM), proline (12 and 24 mM) and SA + proline and developed under the same photoperiodic settings at 4, 8, 16 and 24°C. The maximum increases in proline at the lowest temperature (4°C) and the highest temperature (24°C) were observed to be in 24 mM proline + 0.25 mM SA and 24 mM proline + 0.50 mM SA treatments, respectively. The maximum increases in apoplastic protein at 4, 16 and 24°C were observed to be in co-administration of proline and salicylic acid, whereas the maximum increase in apoplastic protein at 8°C was observed to be in 12 mM proline treatment. Findings indicated that proline and salicylic acid treatment led to the accumulation of proline and apoplastic protein which increased the cold tolerance of pepper callus.

Keywords: Apoplastic protein; Callus; Cold tolerance; Proline; Salicylic acid

Introduction

Tissue culture techniques are preferred in physiological studies and for the purposes of clarifying basic mechanisms since they allow working with a large number of plant materials in small areas under controllable optimum conditions. Previous studies showed that biosynthetic activities of cells can be increased by organizing the characteristics of culture media. *In vitro* tissue culture methods, artificial selection, and stimulation of cells with high level of metabolic activity can be used to increase the amount of compounds produced by the cells significantly and these methods help development of materials with higher tolerance and resistance (Vanisree *et al*., 2004).

Pepper is sensitive to cold and its growth, development, and agricultural productivity decreases when the weather temperature falls below 15ºC. It is an important vegetable among horticultural products in many countries under tropical, subtropical, and temperate climates. Fletcher (1983) and Sanghera *et al.* (2011) reported that cold stress caused far more devastating damage to and greater economic losses of agricultural products of a country than the damage caused by other atmospheric phenomena or sometimes pesticides and diseases. On the other hand, plants try to tolerate the cold stress with different biochemical and physiological compounds.

Exposure to a low temperature usually induces a variety of physiological (soluble proteins, apoplastic protein (having antifreeze activity), proline, enzymes etc.) and molecular changes in plants, which can result in an acclimation response that is characterized by a greater ability to resist injury or survive an otherwise lethal low temperature stress (Levitt, 1980) Synthesis of specific proteins such as apoplastic protein is an important mechanism involved in increasing freezing tolerance during cold acclimation (Antikainen *et al.*, 1996).

Salicylic acid (SA) is a signaling molecule in plants that is required for stimulating specific responses against various biotic and abiotic stresses. Many researchers have suggested that this molecule may be a new plant regulator (Hayat and Ahmad, 2007). However, molecular events included in SA signaling are not yet fully understood; therefore, the studies in this area are increasing.

Proline accumulation is believed to play adaptive roles in plant stress tolerance (Verbruggen and Hermans, 2008). Although much is known about proline metabolism, some aspects of its physiological functions are still lacking, but, the engineering of proline metabolism could lead to new approaches to improve plant tolerance of most important stress (Szabados and Savoure, 2010). Alternative approaches also include the use of proline as an external application, which can also be administered for increasing tolerance to various stresses (Kaya *et al.*, 2007). Some striking evidences of exogenous application of proline and SA to counteract environment stresses are expected to promote their extended application to other plant species.

The aim of this study is to investigate the changes in apoplastic protein and proline content in cold tolerance of

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pepper tissue cultures exposed to proline and salicylic acid treatments at 4, 8, 16 and 24°C.

Material and Methods

Plant Material

The pepper seeds used in this study (*Capsicum annuum* L.) (Kahramanmaras - Line 187) were obtained from Agricultural Research Institute.

Surface Sterilization of Seeds, Germination and Culture Conditions

Pepper seeds (Kahramanmaras - Line 187) were subjected to surface sterilization prior to sowing. For this purpose, pepper seeds were placed inside a glass jar with lid; 70% ethyl alcohol was added onto them and they were kept in ethyl alcohol for 3 min. Ethyl alcohol was filtered and surface sterilization was performed by the addition of 40% commercial sodium hypochlorite. Then the seeds were washed 3 times with sterile distilled water. Seeds subjected to sterilization in this way were sown in medium inside magenta dishes under sterile conditions.

Murashige and Skoog (MS; 1962) hormone-free basic medium containing 3% sucrose and 0.7% agar was basically used for germination of pepper seeds. Pepper seedlings emerging from seeds germinated under aseptic conditions were used as the explant source after a four-week incubation period. For this purpose, hypocotyl explants were cut from each seedling. Initially, cuts were made on the root collar to remove the roots; hypocotyl region was dissected into parts of approximately 15 mm and hypocotyl explant was prepared. MS medium containing 3% sucrose and 0.7% agar and 1.0 mg/L 2,4 D and 0.1 mg/L kinetin was used for extraction of callus from hypocotyl explant (Ellialtioğlu *et al*., 1998). The pH of the medium was set to 5.7. Hypocotyl explants were placed horizontally in MS medium. Callus tissues developed from the hypocotyl explant were grown in a climate chamber for two weeks under a photoperiod of 16 h of light and 8 h of dark. Growing callus tissues were transferred to MS medium inside magenta boxes containing 1.0 mg/L 2,4-D and 0.1 mg/L kinetin, 3% sucrose, and 0.7% agar. Callus tissues in magenta boxes were developed in a climate chamber again under the same photoperiodic settings. Callus tissue developing in magenta boxes including 1.0 mg/L 2,4 - D and 0.1 mg/L kinetin, 3% sucrose and 0.7% agar were transferred to an MS medium containing SA (0.25 and 0.50 mM), proline (12 and 24 mM) and SA + proline. Callus tissues in magenta boxes were again developed under the same photoperiodic settings at 4, 8, 16 and 24°C.

Analysis of Free Proline

The amount of free proline was determined according to the method of Bates *et al.* (1973).

Analysis of Apoplastic Protein

Apoplastic proteins were extracted from pepper callus tissues by vacuum infiltration with 20 mM ascorbate and 20 mM $CaCl_2$ (pH 3), followed by centrifugation at 900 g to recover the apoplastic contents (Hon *et al.*, 1994). Protein concentrations were measured using a modified Bradford procedure with BSA as standart protein (Bradford, 1976).

Statistical Analysis

Callus samples of control and proline, SA and proline + SA treatment were analyzed by applying factorial variance analysis techniques to the data obtained as a result of the analyses (in terms of proline, apoplastic protein), with 3 repetitions (n = 3). Data presented are mean values±standard error of measurement (SEM) for 3 replicates. Data were determined by 2-way ANOVA. Variance analysis was conducted using the SPPS 15 statistical soft ware package.

Results

In our study, 12 and 24 mM proline was administered to the culture medium. At the end of the experiment the maximum callus development was observed to be in 24 mM proline treatment at 24°C (Fig. 1).

In our study, 0.25 and 0.50 mM salicylic acid was administered to the culture medium. When 0.50 mM SA individually administered at 4°C was compared to the control group, it was observed to increase the amount of proline significantly; an increase in proline amount at 8°C was detected in 0.25 and 0.50 mM SA treatments ($P<0.05$) (Fig. 2). However, these concentrations did not show the same effect at 16 and 24°C; when compared to the control group, reductions were observed in the amount of proline ($P<0.05$) (Fig. 2).

When 12 and 24 mM proline-only treatments were compared to the control group at all treatment temperatures, a significant increase was observed in the amount of proline. The maximum increase in the amount of proline was determined in the 24 mM proline treatment at 24°C (Fig. 2).

When SA and proline were co-administered, 24 mM proline + 0.25 mM SA and 24 mM proline + 0.50 mM SA at 4 and 16°C as well as 12 mM proline + 0.50 mM SA, 24 mM proline + 0.25 mM SA, and 24 mM proline + 0.50 mM SA at 8 and 24°C treatments significantly increased the amount of proline as compared to the control ($P<0.05$) (Fig. 2). Among all treatments, the maximum increase in proline amount in comparison to the control group was observed in 24 mM proline + 0.25 mM SA and 24 mM proline + 0.50 mM SA treatments at 24°C ($P<0.05$) (Fig. 2).

When individually administered 0.25 mM SA was compared to the control group, it was observed that it did not result in any significant change in the amount of apoplastic protein. When 0.50 mM SA treatment was compared to the control group at 4 and 16°C, it increased the amount of apoplastic protein ($P<0.05$); however, it did

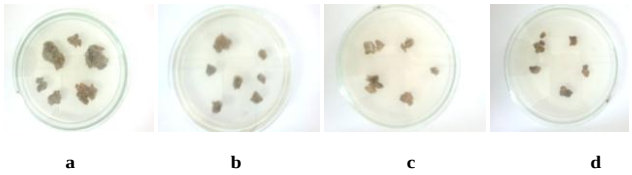

Fig. 1: Callus development in 24 mM proline treatment (a. 24°C, b. 16°C, c. 8°C, d. 4°C)

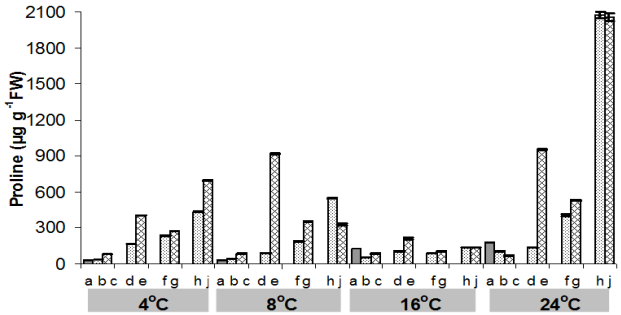

Fig. 2: Effect on proline accumulation of proline and salicylic acid treatment in pepper callus at 4, 8, 16 and 24 °C. (a: Control, b: 0.25 mM SA, c: 0.50 mM SA, d: 12 mM proline, e: 24 mM proline, f: 0.25 mM SA + 12 mM proline, g: 0.50 mM SA + 24 mM proline, h: 0.25 mM SA + 24 mM proline, j: 0,50 mM SA + 24 mM proline)

not have the same effect at 8°C and 24°C (Fig. 3).

When individually administered 12 mM proline treatment was compared to the control group, it was observed that it increased the amount of apoplastic protein ($P< 0.05$); however, it did not cause a significant change at 8 and 16°C. On the other hand, a slight decrease was detected in the amount of apoplastic protein at 24°C (Fig. 3).

When SA and proline were co-administered and compared to the control group, the maximum increases in apoplastic protein were observed in 12 mM proline + 0.25 mM SA and 12 mM proline + 0.50 mM SA treatments at 16°C and 24 mM proline + 0.25 mM SA and 24 mM proline+0.50 mM SA treatments at 24°C ($P< 0.05$) (Fig. 3).

Discussion

Low temperature is one of the most important stress factors limiting plant growth and development (Janda *et al*., 2003). Low temperature leads to damages such as dehydration, decreases in functions of biomembranes, inactivation of membrane-bound ion pumps, and oxidative stress, etc. These damages caused by low temperature differ depending on the developmental phase, the severity of cold, and ice formations in intracellular or extracellular spaces (Beck *et al*., 2004).

Plants form various compounds to protect cells against intracellular and intercellular ice formations. Many plants accumulate compounds such as (extracellular) proteins, amino acids, and sugars in the apoplastic region (Griffith and Yaish, 2004).

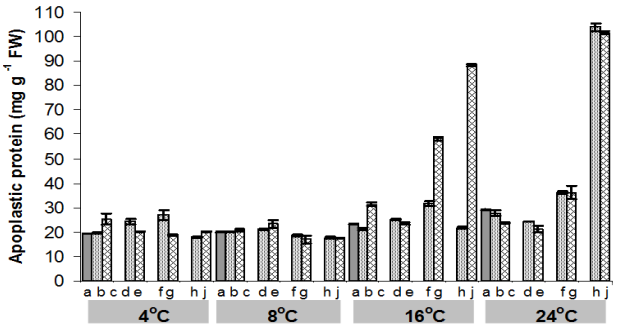

Fig. 3: Effect on apoplastic protein of proline and salicylic acid treatment in pepper callus at 4, 8, 16 and 24 °C. (a: Control, b: 0.25 mM SA, c: 0,50 mM SA, d: 12mM proline, e: 24 mM proline, f: 0,25 mM SA + 12mM proline, g: 0,50 mM SA + 24 mM proline, h: 0,25 mM SA + 24 mM proline, j: 0,50 mM SA + 24 mM proline)

Proline is an osmoprotectant, and is considered as most common physiological response generated against biotic and abiotic stresses (Kavıanı, 2007). Smirnoff and Cumbes (1989) suggested in their study that proline could be a ROS catabolizer. They advocated that this protective role of proline might be related to its antioxidant feature. Rasheed *et al.* (2010) reported that increased of proline amount under chilling stress. In this study we also determined that proline accumulation was a typical abiotic stress response. Our findings suggested that at low temperatures (4 and 8ºC), externally administered proline, either individually or together with salicylic acid, significantly increased the endogenous proline content in comparison to the control group. The evidences in this study demonstrate that the addition of 12 and 24 mM proline to culture medium resulted in increase in endogenous proline content. In this study, we determined that the most effective concentration of exogenous proline in culture medium was 24 mM proline. Our results suggested that exogenously applied proline might be act as a signaling molecule and may stimulate defense pathways and endogenous proline biosynthesis.

Under various stress conditions, the antioxidant capacity may not be sufficient to reduce the effect of oxidative damage. For this reason, signaling molecules should also be studied in order to understand how the plant under stress develops its stress tolerance. Recent studies have shown that SA is a natural, hormone-like and at the same time phenolic signaling molecule necessary for the activation of plant defense system (Klessig and Malamy, 1994).

In their study on tomato and bean Senaratna *et al.* (2000) determined that the tolerance of plants grown from 0.1 - 0.5 mM SA-administered seeds increased against heat, frost, and drought stress. In a similar study, Tasgin *et al.* (2003) studied winter wheat and determined that the frost damage to plants was lower in 0.1 and 1 mM SA treatments under controlled conditions.

Chen and Chen (1999) determined that when salicylic acid was individually administered on *transforme salvia*

miltiorrhiza cell suspension cultures, it did not prod the stimulation of phytoalexin. They identified that salicylic acid was effective when used in conjunction with another stimulant. In our study, 0.25 and 0.50 mM salicylic acid was administered to the culture medium. Individually administered salicylic acid concentrations increased the amount of proline in comparison to the control group at low temperatures (4 and 8°C) and this results indicated that SA induced chilling tolerance; however, they did not have the same effect at 16°C and 24°C. Our results suggested that SA can protects pepper callus against chilling stress by increasing the proline accumulation. On the other hand, when SA and proline were co-administered, they had a positive effect generally at all temperatures. This proline increase may be due to SA's acceleration of plant metabolism. From data of this study, it can be concluded that the treatment of SA and proline may protect pepper callus cultures generally against chilling stress.

The effects of biotic and abiotic stress on apoplastic region are studied by researchers. Apoplastic regions were reported to be probably important in plant cells' response to stress (Minibaeva *et al*., 2001). Therefore, the effect of SA and cold on apoplastic protein in pepper tissue culture was investigated in this study. Tasgin *et al*. (2006) reported in their study on winter wheat leaves that SA and cold treatment might increase cold tolerance by increasing the accumulation of apoplastic proteins, which included antifreeze proteins and prevented formation of ice crystals. They determined that SA had an important role in regulation of apoplastic proteins and activation of antioxidant defense system. In our study, 0.50 mM SA treatment increased the amount of apoplastic protein at 4 and 16°C; however, more significant changes were detected when SA and proline were co-administered. In this study, the most effective concentrations of proline+SA (co-administered) in culture medium were 24 mM proline+0.25 mM SA and 24 mM proline+0.50 mM SA, which increased endogenous proline and apoplastic protein accumulation. We found a significant correlation between apoplastic protein level and increase of proline content in of pepper callus cultures after exposure to 24 mM proline +0.50 mM SA treatments at 24°C. Low and high level of SA and the temperature of treatment affect the tolerance of pepper callus against cold stress.

The type of plant, stress factor, concentration of stress factor, duration of exposure to stress, and the structure of the tissue or organ exposed to stress are also effective in this process. From the results of this study and the previous studies demonstrate the necessity of many more research studies on SA (different concentrations, applied method etc.) and proline signaling in plants.

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