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Full Length Research Paper

The effects of exogenous proline and osmotic stress on morpho-biochemical parameters of strawberry callus

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For evaluation of growth parameters of strawberry callus under osmotic stress and exogenous proline, embryonic calli were transferred to Murashige and Skoog (MS) medium containing four sucrose (osmotic stress) treatments including 3, 6, 9 and 12% and various concentrations of exogenous L-proline (0, 2.5, 5 and 10 mM) supplemented with 1 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.5 mg/l 6-benzyl amino purine (BAP). After four weeks, various morpho-biochemical parameters of calli were studied. Callus fresh and dry weight was strictly dependent on the concentration of sucrose in the medium. When the concentration of sucrose in the medium was increased from 3 to 9%, the amount of fresh weight was decreased, while dry weights increased in callus tissues. High concentration of sucrose (12%) caused the least content of fresh and dry weight in calli. When the concentration of sucrose in the medium was increased from 3 to 12%, the amount of free proline of callus was increased. Addition of exogenous proline to the culture medium improved the growth of calluses and intracellular free proline. The highest content of fresh weight (601.09 mg), dry weight (134.68 mg) and content of proline accumulation (11.12 µMoles/g fresh weight) were obtained in calli grown on media containing 10 mM proline, while the least content of fresh weight (297.62 mg), dry weight (49.55 mg) and free proline accumulation (0.817 µMoles/g fresh weight) were observed in calli of control (without proline).

Key words: Strawberry, proline, sucrose, osmotic stress, fresh weight, dry weight, callus.

INTRODUCTION

Strawberry is a perennial, stoloniferous herb that belongs to the family *Rosaceae*, genus *Fragaria* and most widely consumed fruits throughout the world. Callus cultures are used as an *in vitro* technique for biochemical and physiological studies in response to stress at the cellular level (Basu et al., 2002; Jantaro et al., 2003; Liu et al., 2006). Low sucrose concentration (3%) is much more effective than high levels on callus fresh weight. The rise of sucrose concentration decrease callus fresh weight and this capacity by the increase of sucrose concentration could be attributed to an osmotic effect (Gerdakaneh et al., 2009). Sucrose in culture medium functions both as a carbon source and osmotic regulator. Both functions are critical for embryogenesis and callus formation (Last and Brettell, 1990). Osmotic stress affects synthesis of proline and has been suggested as one of the possible means for overcoming osmotic stress.

Proline has been proposed to act as a compatible solute that adjusts the osmotic potential in the cytoplasm (Arshi et al., 2005; Caballero et al., 2005; Bartels and Sunkar, 2006). It is considered to play an important role in defense mechanisms of stressed cells. In view of Serraj and Sinclair (2002) osmotic adjustment is one of the major physiological phenomena vital for sustaining growth of plants under osmotic stress. Proline accumulation in higher plants is a characteristic physiological response to osmotic stress. Its degradation can provide carbon, nitrogen and energy source after stress (Hare et al., 1999).

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Abbreviations: MS, Murashige and Skoog; **BA,** 6-benzyladenine; **GA3,** gibberellic acid; **IBA,** indole–3- butyric acid; **NAA,** naphthaleneacetic acid; **BAP,** 6-benzyl amino purine; **2,4-D,** 2,4-dichlorophenoxy acetic acid.

Table 1. Effect of exogenous proline and different sucrose concentrations on fresh weight (mg) of embryonic calls in the leaf explant of strawberry cv. Kurdistan.

Sucrose (%)	Proline rate (mM)				
	Initial (mg)	0	2.5	5	10
3.0	100 ± 2	437.53h	478.49g	527.64e	552.64d
6.0	100 ± 2	355.08j	545.21de	587.52c	636.74b
9.0	100 ± 2	198.93k	489.79fg	602.11c	673.99a
12.0	100 ± 2	159.08l	401.45i	499.71f	540.97de
Average		297.62	478.74	554.25	601.09

Means with the same small letter in columns do not differ significantly by Duncan's multiple range tests ($p < 0.05$).

As there is no information on link between osmotic stress, exogenous proline and callus growth of strawberry, this study is aimed at clarifying the relationship between osmotic stress, exogenous proline and morpho-biochemical aspects in strawberry callus tissues.

MATERIALS AND METHODS

The experiment was conducted to study *in vitro* assessment of sucrose induced osmotic stress and effects of proline in callus tissues of strawberry. For this purpose, runner tips of Kurdistan cultivar were taken from greenhouse and washed in running tap and then explants were surface-sterilized by a 10 s immersion in 70% (v/v) ethanol and for 15 min in aqueous solution of 1% (v/v) sodium hypochlorite. After three washes in sterile double distilled water, sterilized runner tips were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 0.5 mg/l 6-benzyladenine (BA), 0.1 mg/l gibberellic acid (GA3) and 0.1 mg/l indole–3- butyric acid (IBA), as described by Boxus (1999). The pH of medium was adjusted to 5.8 using 0.1N NaOH or HCl and the medium was solidified with 0.8% agar before autoclaving. The cultures were incubated in a 16-h photoperiod under 35 µmol m^{-2} s^{-1} illuminations in cool, white fluorescent light at 25 ± 1°C. Pieces from fully expended young leaves of 4 - 5 week-old plantlets from auxiliary shoots of *in vitro* cultured runner tips segments (each approximately 4 × 3 mm) were used as explants.

Callus induction

Explants were cultured on MS medium supplemented with 4 mg/l naphthaleneacetic acid (NAA) for callus induction. 6 - 8 explants were cultured in each Petri dish (100 X 20 mm) containing 30 ml of medium. During callus induction period, all the cultures were incubated in the dark at 25 ± 1°C.

Sucrose and exogenous proline treatments

For evaluation of growth parameter under osmotic stress and exogenous proline, calli produced were transferred to medium containing four sucrose (osmotic stress) treatments including control (control (3%), 6, 9 and 12%) and 0, 2.5, 5 and 10 mM L-proline. About 100 mg (fresh weight) calli were cultured in each Petri dish (100 X 20 mm) containing 30 ml of medium supplemented with the above mentioned sucrose and L- proline concentrations supplemented with 1 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D) + 0.5 mg/l 6-benzyl amino purine (BAP). After four weeks, calli were harvested and various morpho-biochemical parameters were studied. For estimation of fresh weight, dry weight and proline content, calli after four weeks of treatments harvested from medium, were first washed with deionized distilled water for 2 min. After being dried with tissue paper, fresh weights of calli were determined. Callus dry weights were determined after calli were dried in oven at 65°C for 72 h. Extraction and measurement of free proline content was conducted according to the procedure described by Bates et al. (1973). Factorial analysis of variance was carried out using statistical analysis system (SAS) and all data were analyzed according to Duncan (1955) test.

RESULTS AND DISCUSSION

This investigation on strawberry explores the effect of osmotic stress and exogenous proline on callus growth rate and endogenous proline content. Calli were cultured on MS medium supplemented with 1 mg/l 2,4-D + 0.5 mg/l BAP, different concentrations of sucrose (3, 6, 9 and 12%) and various concentrations of proline (0, 2.5, 5 and 10 mM).

The results after four weeks indicated that sucrose concentration in the medium significantly affected callus growth. Callus fresh and dry weights were strictly dependent on the concentration of sucrose in the medium. Sucrose (3 to 9%) induced osmotic stress with regard to callus growth rate (fresh) revealed that increasing concentration of sucrose in culture medium showed a significant decline (Table 1), while dry weights increased significantly in callus tissues (Table 2). The differences in frequencies of callus fresh and dry weights between sucrose concentrations were statistically significant ($P < 0.0005$). *In vitro* addition of sucrose in the growth medium acts as osmotic agent that may introduce osmotic stress above certain concentrations and then lead to decrease in growth (Mehta et al., 2000; Kim and Kim, 2002), while dry weights increased under increasing sucrose osmotic stress (Kishore and Dange, 1990; Juhasz et al., 1997). The increase in dry weight of callus tissue was due to the more accumulation of proline and total soluble carbohydrates in the callus tissues. Sucrose is very important for the culture of embryonic calli because besides being a carbon source, they regulate medium osmotality, a critical

Table 2. Effect of different sucrose concentrations and exogenous proline on dry weight (mg) of embryonic culture in the leaf explant of strawberry cv. Kurdistan.

Sucrose (%)	Proline rate (mM)			
	0	2.5	5	10
3.0	68.73ij	81.29h	90.81gh	95.94fg
6.0	65.22j	118.15de	131.78c	144.98b
9.0	41.44k	115.05de	150.59b	173.75a
12.0	22.81l	79.53hi	106.03ef	124.05cd
Average	49.55	98.51	119.80	134.68

Means with the same small letter in columns do not differ significantly by Duncan's multiple range tests ($p < 0.05$).

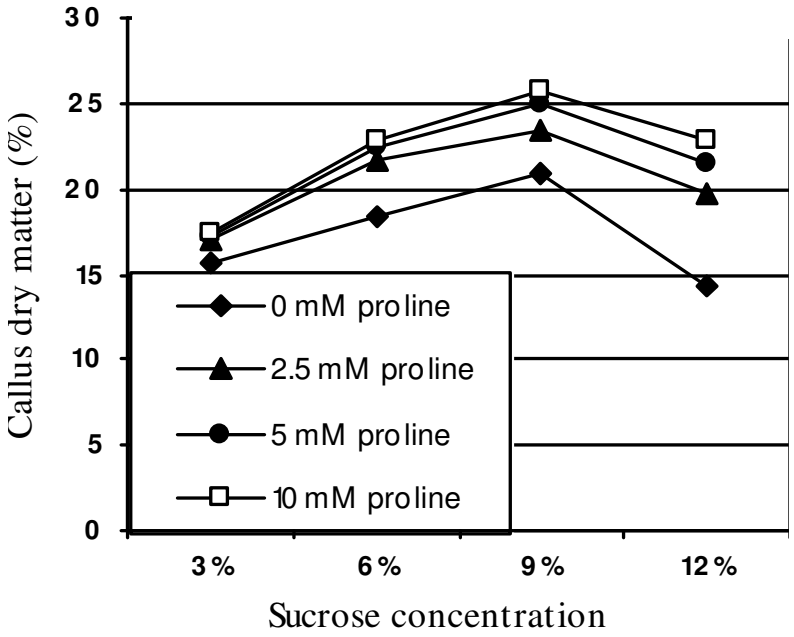

Figure 1. Effect of exogenous proline and different sucrose concentrations on percentage of embryonic callus dry matter in the leaf explant of strawberry cv. Kurdistan.

factor in embryonic calli development *in vitro*. In addition to this, some others (Carrier et al., 1997; Johnson et al., 1997) reported that sucrose might play multiple roles including the prevision of carbon and energy, causing an osmotic effect (Figure 1).

The addition of high concentration of sucrose (12%) to the culture medium caused a decline in the growth of calluses. On MS supplied with high sucrose concentration, the growth of callus was completely inhibited due to abundant callus formation. The least content of fresh weight and dry weight in calli treated with 12% sucrose (without proline) established that the highest levels of sucrose caused the highest stress and the highest stress caused the least content of fresh weight and dry weight in calli (Tables 1 and 2). Osmotic potential of the calli increased with increasing stress level of the culture medium (Bajji et al., 2000; Huang and Liu, 2002), particularly by sucrose (Riham et al., 2001).

After four weeks, the endogenous proline content of explants treated with different concentrations of sucrose, increased with increasing concentration of the treatment, thus, corroborating the role of proline as an osmolyte during stress conditions. Free proline significantly increased in callus tissues as the sucrose concentration increased to culture medium (Table 3). When the concentration of sucrose in the medium was increased from 3 to 12%, the amount of free proline inside of the cells was increased. However, there was a significant difference among the results in the calluses cultured in different medium with sucrose. The results showed that significant differences

Table 3. Effect of exogenous proline and different sucrose concentrations on intracellular free proline (µmoles/g fresh weight) of embryonic callus in the leaf explant of strawberry cv. Kurdistan.

Sucrose (%)	Proline rate (mM)			
	0	2.5	5	10
3.0	0.061l	2.918i	4.822g	7.242e
6.0	0.382kl	4.223h	6.369f	9.891c
9.0	0.856k	4.938g	7.873d	11.955b
12.0	1.969j	6.478f	9.616c	15.380a
Average	0.817	4.64	7.17	11.12

Means with the same small letter in columns do not differ significantly by Duncan's multiple range tests ($p < 0.05$).

were found between different levels of proline of calli treated with the highest level of sucrose showed that proline plays a role in water equilibrium. The highest content of proline accumulation (1.969 µMoles/g fresh weight) was in calli grown on media with the highest level of sucrose (12%). The least content of proline accumulation (0.061µMoles/g fresh weight) was observed in calli of control (sucrose 3%) (Table 3). Analysis of data showed that the difference between proline in cultured calluses at 12% sucrose and the control was very high. The present study revealed that sucrose induced osmotic stress increased free proline in callus tissues (Al-Khayri and Al-Bahrany, 2002). Free proline accumulation increased many folds upon exposure to abiotic stresses (Javed, 2002b; Ahmad et al., 2006).

This study revealed that fresh and dry weights increased in calli of strawberry grown on media supplemented with exogenous proline. Results showed that there was a very highly significant effect of proline and sucrose concentration and of the interaction between them ($p < 0.0005$), with a very highly significant effect of proline at each concentration. Tables 1 and 2 show that when exogenous proline (2.5, 5 and 10mM) and sucrose (3, 6, 9 and 12%) were applied to the medium, the fresh and dry weights of calli increased. Cultures grown on 12% sucrose without exogenous proline turned brown within 14 days indicating necrosis. Cultures grown on 12% with exogenous proline did not turn brown and morphologically resembled those grown on 3% sucrose. Our studies showed that not only can cultures survive high sucrose concentration, but that they are capable of active growth while the osmotic stress is still being applied. Proline aids in the survival of calli. The role of proline in adaptation and survival of plants had been observed by several researchers (Porgali and Yurekli, 2005; Arshi et al., 2005; Djanaguiraman et al., 2006). Osmotic adjustment through the accumulation of cellular solutes, such as proline has been suggested as one of the possible means of overcoming osmotic stress caused by loss of water (Shankhadhar et al., 2000). Our present study exhibited similar results under sucrose induced osmotic stress and exogenous proline, because proline contents of callus tissues increased.

Addition of exogenous proline to the culture medium improved intracellular free proline in response to exogenous proline. After 4 weeks post culture, the concentration of the free proline in the calluses were measured, and the results showed that the calluses in cultures with 2.5, 5 and 10 mM proline showed higher amount of free proline than control (culture with no exogenous proline). Addition of exogenous proline in medium increased the amount of free proline; proline from high concentration was higher than low concentration under exogenous proline associated with stress (Table 3).

In conclusion, callus fresh and dry weight as well as accumulation of free proline was strictly dependent on the concentration of sucrose in the medium and addition of exogenous proline to the culture medium improved the growth of callus and intracellular free proline.

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