

INDUCING SALT TOLERANCE IN CANOLA (*BRASSICA NAPUS* L.) BY EXOGENOUS APPLICATION OF GLYCINEBETAINE AND PROLINE: RESPONSE AT THE INITIAL GROWTH STAGES

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Abstract

In order to assess whether exogenous application of glycinebetaine and proline could induce salt tolerance in canola, two canola cultivars Dunkled (salt tolerant) and Cyclone (salt sensitive) were allowed to germinate and grow at 0, and 120 mM NaCl for two weeks. Varying concentrations (0, 0.1, 0.5, 1 and 5 mM) of glycinebetaine (GB) or proline were applied through the rooting medium to non-stressed and salt stressed germinating seeds. Salt stress caused a marked inhibitory effect on germination and seedling growth of both canola cultivars. This adverse effect of salt stress on seed germination percentage was more on cv. Cyclone than on cv. Dunkled. Exogenously applied GB and proline alleviated the adverse effects of salt stress on seed germination of both canola cultivars. Moreover, salt-induced reduction in seedling growth of both canola cultivars was counteracted by high concentration of GB (1 or 5 mM GB) added to the growth medium. Similarly, considerable growth improvement in salinized seedlings of both canola cultivars was observed when 1 or 5 mM proline was added to the growth medium.

Introduction

Salinity is one of the major abiotic stresses in arid and semi-arid regions that substantially reduce the average yield of major crops by more than 50% (Bray, 2000). These losses are of great concern for most countries, like Pakistan the economy of which rely mainly on agriculture (Athar & Ashraf, 2009). Although salt stress affects all growth stages of a plant, seed germination and seedling growth stages are known to be more sensitive in most plant species (Ashraf, 1994; Munns, 2002; Cuartero *et al.*, 2006). Furthermore, germination and seedling stage is predictive of plant growth responses to salinity (Blum, 1985; Cuartero *et al.*, 2006). Therefore, seeds with more rapid germination under salt stress and/or normal conditions may be expected to achieve a rapid seedling establishment and more salt tolerance, resulting in good stand establishment and hence higher yields (Munns, 2002).

Canola (*Brassica napus* L.) grown mainly for edible oil purpose is a moderately salt tolerant crop (Ashraf & McNeilly, 1990; Francois, 1994). In view of increasing awareness of the health advantages of canola oil and existing salt tolerance potential, its demand has undoubtedly increased during the last two decades. This has resulted into increased cultivation of canola on soils where salinity problems already exist. Thus, there is a need for further improvement in the salt tolerance of canola (Francois, 1994; Steppuhn *et al.*, 2001; Ashraf & McNeilly, 2004). In order to improve crop salt tolerance that will result into enhanced productivity on salt affected soils, different perspective strategies have been proposed by various plant scientists (Ashraf *et al.*, 2008). Of them, exogenous use of compatible organic solutes has gained a considerable ground as a

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shotgun approach to ameliorate the adverse effects of salt stress on plants (Ashraf & Foolad, 2007). For instance, foliar spray of compatible organic solutes such as glycinebetaine (GB) or proline counteracted the growth inhibition induced by NaCl in different crop plants e.g., wheat (Raza *et al.*, 2006), tomato (Makela *et al.*, 1998), rape (Makela *et al.*, 1999) and maize (Ali *et al.*, 2007). Thus, exogenous application of GB or proline improves the growth, survival, and tolerance of a wide variety of plants under various stress conditions (Ashraf & Foolad, 2007). However, the information regarding the role of exogenous GB and proline on germination and early seedling growth is scarce. Hence, the present study was conducted to assess the influence of exogenously applied GB and proline in inducing salt stress tolerance of canola at germination and early seedling growth stages.

Materials and Methods

Two potential compatible solutes glycinebetaine (GB) and proline were applied exogenously through the rooting medium at the germination and seedling stages, to assess up to what extent the application of these compatible solutes could alleviate the adverse effects of salt stress on growth of a potential oil-seed crop canola (*Brassica napus* L.). The seedling experiment was conducted in two sub-experiments i) effect of varying concentrations of glycinebetaine on the germination and seedling growth of two canola cultivars (Dunkled, salt tolerant; Cyclone, salt sensitive) differing in salinity tolerance, and ii) effect of varying concentrations of proline on the germination and seedling growth of two canola cultivars differing in salinity tolerance. Both these sub-experiments were arranged in a completely randomized design with four replicates, two NaCl levels (0 and 120 mM), and five GB or proline levels (0, 0.01, 0.05, 0.1 and 1 mM). For the preparation of solutions of different concentrations (0, 0.01, 0.05, 0.1 and 1 mM) of compatible solutes, glycinebetaine (Mol. wt = 117.15) and proline (Mol wt = 115.13) of Sigma Aldrich, Germany were prepared in distilled water. From both these experiments, it was evident that salt stress reduced the germination percentage, speed of germination and seedling growth of both canola cultivars. However, exogenous application of both GB and proline enhanced the total germination percentage, and seedling growth. Moreover, this counteractive effect of both GB and proline on canola cultivars at the seedling stage was maximal at the highest GB or proline level (0.1 mM) applied in the rooting medium. In view of these results, two more sub-experiments were conducted in a similar way but concentration of compatible solutes was increased up to 5 mM, i.e., (0, 0.1, 0.5, 1 and 5 mM) to assess the upper toxic limit of compatible solutes. A brief description of these sub-experiments is given below:

Seeds of the salt tolerant cultivar Dunkled and the salt sensitive Cyclone of canola (*Brassica napus* L.) were surface sterilized in 5% Sodium hypochlorite solution for 5 minutes before further experimentation. Two hundred seeds (50 seed per Petri plate) of each cultivar were placed in Petri plates (18 cm diameter) double lined with filter paper moistened with 10 ml of different concentrations (0, 0.1, 0.5, 1 and 5 mM) of GB or proline added in full strength Hoagland's nutrient solution containing 0 or 120 mM NaCl. All the seeds (in a set of 80 Petri plates of each sub experiment) were then allowed to germinate for 8 days in a growth room (PAR, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$; temperature 25°C \pm 2°C and relative humidity [RH], 58-70%). Germination rate and total germination percentage were recorded for 8 days, after which time the seedlings were harvested. Shoot and root of each seedling were separated and data for fresh biomass recorded. These plant parts were then oven-dried at 65°C for 72 h and dry biomass recorded.

Table 1. Mean squares from analysis of variance (ANOVA) of the data for germination percentage at different day's intervals of two canola (*Brassica napus* L.) cultivars differing in salinity tolerance when different levels of GB were exogenously applied to salt stressed or non-stressed plants at the seedling stage.

Source of variation	df	Germination % at day 2	Germination % at day 4	Germination % at day 6	Germination % at day 8
Salt	1	6450.03***	10888.8***	4856.8***	1516.7***
Cvs	1	1.70ns	605***	245***	5.8ns
GB	4	93.09***	150.2***	836**	29.6ns
Salt x Cvs	1	146.70***	1003.4***	190.1**	76.7*
Salt x GB	4	16.87*	5.6ns	48.3*	16.2ns
Cvs x GB	4	9.86ns	12.3ns	22.5ns	3.0ns
Salt x Cvs x GB	4	12.32*	2.5ns	31.3ns	36.5*
Error	60	4.68	27.1	18.5	13.3

ns = Non-significant; *, **, *** = Significant at 0.05, 0.01 and 0.001 probability

GB = Glycinebetaine, Salt = Salt stress; Cvs = Cultivars

Statistical analysis: The experiments were set up in a completely randomized design (CRD) with four replicates. Analysis of variance of all parameters was computed using the CoStat v 6.3 computer package. The least significance difference between the mean values was calculated following Snedecor & Cochran (1980).

Results

Analysis of variance of the data for germination percentage of canola cultivars shows that germination percentage of both cultivars decreased ($p \leq 0.001$) significantly due to the addition of salt to the rooting medium (Table 1; Fig. 1). Cultivars also differed significantly and cv. Dunkeld exhibited higher seed germination percentage at all day intervals except at day 8 of salinity exposure where both canola cultivars exhibited similar germination percentage under saline conditions (Fig. 1). Exogenous application of glycinebetaine (GB) through the rooting medium enhanced the germination percentage of both cultivars. However, this improving effect was more in salt stressed plants than that in non-stressed plants due to the addition of 1 or 5 mM GB to the rooting medium after 8 days of exposure to salt stress (Fig. 1).

Fresh and dry weights of shoots and roots of both canola cultivars markedly decreased due to salt stress. Canola cultivars differed significantly ($p < 0.05$) in shoot dry weight only. However, exogenous application of varying concentrations of glycinebetaine significantly ($p \leq 0.01$) enhanced the shoot dry weight, and root fresh and dry weights of both cultivars under saline conditions. But maximum improvement in terms of shoot dry weight was recorded at 1 or 5 mM GB under saline conditions (Table 2; Fig. 2). A significant improvement in root fresh weight of salt stressed plants of both canola cultivars occurred due to exogenously applied 5 mM GB, while this was true for root dry weight under non-saline conditions (Fig. 2).

Data for fresh and dry weights of shoots and roots of both canola cultivars show that there was a significant inhibitory effect ($p \leq 0.001$) of the addition of NaCl to the growth medium on these growth attributes (Table 3). Canola cultivars differed significantly ($p < 0.01$) only in terms of shoot dry weight. Incubation of canola seedlings in different concentrations of proline significantly ($p \leq 0.001$) increased the shoot and root dry weights of both cultivars under saline conditions. However, a maximum increase in dry weight of shoots and roots of salt stressed plants of both canola cultivars was recorded at 5 mM proline (Fig. 3).

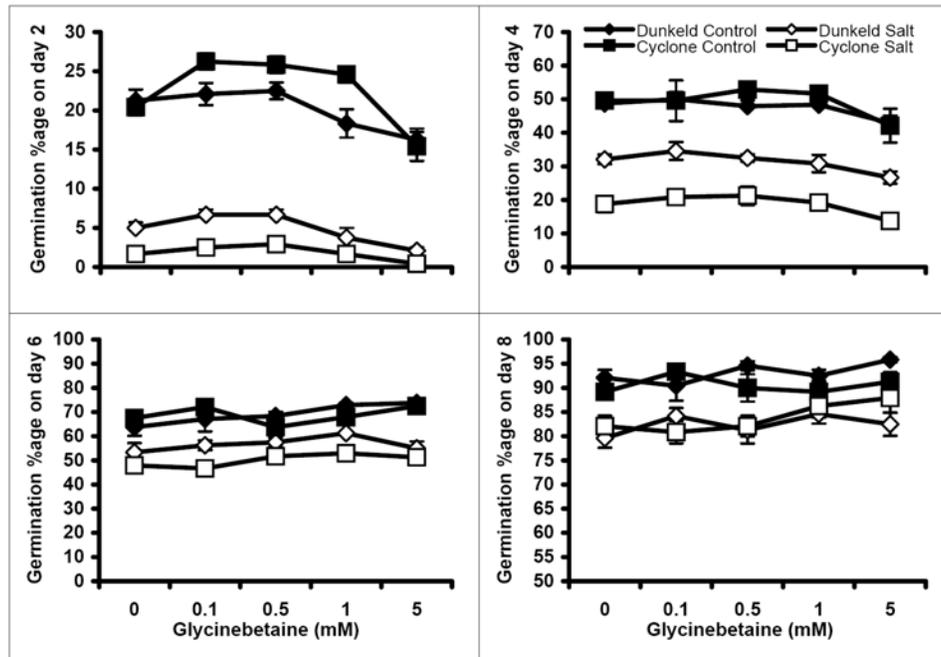


Fig. 1. Germination percentage at different day's intervals of two canola (*Brassica napus* L.) cultivars differing in salinity tolerance when different levels of GB were exogenously applied to salt stressed or non-stressed plants at the seedling stage.

Table 2. Mean squares from analysis of variance (ANOVA) of the data for shoot and root fresh and dry weights of two canola (*Brassica napus* L.) cultivars differing in salinity tolerance when different levels of GB were exogenously applied to salt stressed or non-stressed plants at the seedling stage.

Source of variation	df	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight
Salt	1	870.38***	6.866***	492.83***	2.62***
Cvs	1	57.25ns	0.732*	0.075ns	0.01ns
GB	4	52.49ns	0.770***	7.71**	0.13***
Salt x Cvs	1	2.10ns	0.017ns	4.42ns	4.69e-5ns
Salt x GB	4	35.39ns	0.040ns	1.16ns	0.011ns
Cvs x GB	4	27.53ns	0.021ns	0.85ns	0.010ns
Salt x Cvs x GB	4	5.09ns	0.01ns	1.53ns	0.004ns
Error	60	32.66	0.116	2.04	0.006

ns = Non-significant; * ** *** = Significant at 0.05, 0.01 and 0.001 probability

GB = Glycinebetaine, Salt = Salt stress; Cvs = Cultivars

Discussion

Germination and seedling establishment are very sensitive growth stages of several plant species. It is well evident that a crop species or cultivar with better germination and seedling growth under salt stress will be more stress tolerant at later stages and will produce better crop growth and productivity (Francois, 1994; Ashraf & McNeilly, 2004; Ahmadi & Arkedani, 2006). In the present study, a marked inhibitory effect of salt stress on germination of both canola cultivars at different intervals was observed. However, this

adverse effect of salt stress on seed germination percentage was more on cv. Cyclone than that on cv. Dunkeld. From the mean data for seed germination percentage at different intervals, it is evident that salt stress also caused a more delay in seed germination of cv. Cyclone than in cv. Dunkeld (Fig. 1). Addition of 0.1 or 0.5 mM GB in the growth medium improved the germination percentage of both cultivars after two days of salinization, whereas 1 or 5 mM GB did not prevent the adverse effects of salt stress on germinability of canola seeds. However, addition of 1 or 5 mM GB in the growth medium caused an increase in seed germination percentage in both cultivars after eight days of salinization. Moreover, salt-induced reduction in seedling growth of both canola cultivars was counteracted by higher concentration of GB (1 or 5 mM GB) added to the growth medium (Fig. 2). Similarly, considerable growth improvement in salinized seedlings of both canola cultivars was observed when 1 or 5 mM proline added to the growth medium as reflected from data for shoot fresh and dry weights (Fig. 3). These findings can be related to some earlier studies in which it has been observed that exogenous application of glycinebetaine or proline counteracts the growth inhibition caused by salt stress or cold stress in different crop plants (Roy *et al.*, 1993; Naidu & William, 2004; Cha-um *et al.*, 2006; Posmyk & Janas, 2007). For example, Bal (1976) reported that seed treatment with proline induced salt tolerance in *Brassica napus*. In rice, exogenous application of 30 mM proline counteracted the adverse effects of salinity on early seedling growth (Roy *et al.*, 1993). Likewise, in rice seedlings, exogenous application of GB increased the salt-tolerance of the seedlings by improving plant water status (Cha-um *et al.*, 2006). Recently, Ueda *et al.*, (2007) reported that salt induced reduction in growth of wild type and transgenic plants of *Arabidopsis thaliana* expressing *HvProT* was recovered by 0.1 or 1 mM proline added to the growth medium.

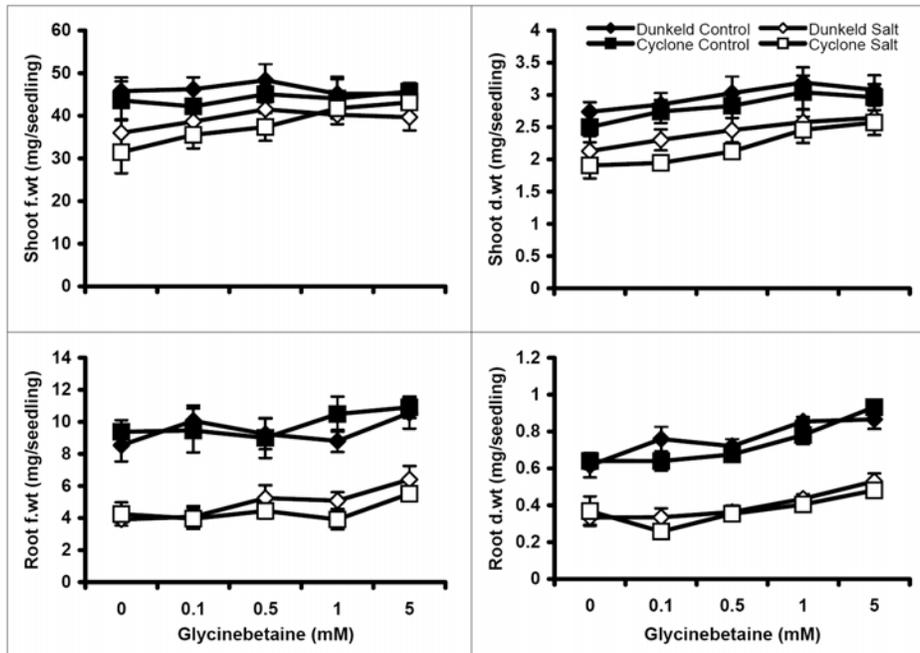


Fig. 2. Shoot and root fresh and dry weights of two canola (*Brassica napus* L.) cultivars differing in salinity tolerance when different levels of GB were exogenously applied to salt stressed or non-stressed plants at the seedling stage.

Table 3. Mean squares from analysis of variance (ANOVA) of the data for fresh and dry weights of shoot and root of two canola (*Brassica napus* L.) cultivars differing in salinity tolerance when different levels of proline were exogenously applied to salt stressed or non-stressed plants at the seedling stage.

Source of variation	df	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight
Salt	1	825.5***	8.42***	756.85***	2.98***
Cvs	1	42.0ns	0.56**	4.33ns	0.03ns
Proline	4	33.7ns	1.23***	3.63ns	0.12***
Salt x Cvs	1	70.4ns	0.05ns	1.94ns	1.85e-4ns
Salt x Proline	4	3.3ns	0.10ns	3.50ns	0.006ns
Cvs x Proline	4	1.2ns	0.07ns	1.95ns	0.003ns
Salt x Cvs x Proline	4	8.0ns	0.004ns	0.84ns	0.006ns
Error	60	19.90779	0.051	2.762818.3	0.012

ns = Non-significant; **,** = Significant at 0.01 and 0.001 probability

Salt = Salt stress; Cvs = Cultivars

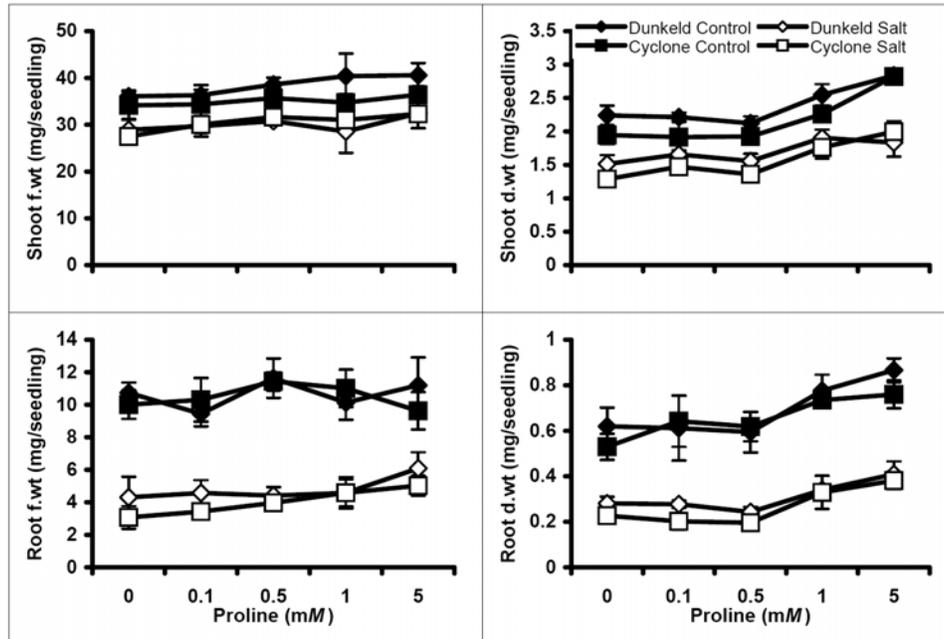


Fig. 3. Fresh and dry weights of shoot and root of two canola (*Brassica napus* L.) cultivars differing in salinity tolerance when different levels of proline were exogenously applied to salt stressed or non-stressed plants at the seedling stage.

It is well evident that salt-induced reduction in seed germination and early seedling growth is due to low availability of water, and changes in the activities of certain enzyme due to intake of toxic ions (Filho & Sodek, 1988; Guerrier, 1988), and due to reduction in hydrolysis and utilization of food reserves (Ahmad & Bano, 1992; Mondal *et al.*, 1988). In view of the results of the present study that exogenous application of GB or proline improved the seed germination and early seedling growth of both canola cultivars under saline conditions, it is likely that both compatible solutes (GB and proline) might have been absorbed by the developing seedlings though not measured here, where they might

have maintained a better water status by increasing water influx and reducing efflux of water under salt-induced water limited conditions (Rahman *et al.*, 2002; Kavi Kishore *et al.*, 2005; Chen & Murata, 2008), they might have protected the membranes against ion toxicity and salt-induced oxidative stress in germinating seeds (Mansour, 1998; Sivakumar *et al.*, 2000; Chen & Murata, 2008), increased the cellular growth (Kumar & Sharma, 1989; Banu *et al.*, 2009) and thus increased the seedling growth of canola.

It is also evident from the results of the present study that early growth inhibitory effect of salt stress was more on cv. Cyclone than on cv. Dunkeld. This difference in growth of both canola cultivars under saline conditions was expected in view of differential genetic potential of these two cultivars for salinity tolerance. Cultivar Dunkeld is known for its high salt tolerance (Qasim *et al.*, 2003; Ashraf & Ali, 2008), and Cyclone is a moderately salt sensitive (Qasim *et al.*, 2003; Ulfat *et al.*, 2007).

In conclusion, exogenous applications of GB and proline at the germination and seedling stages alleviated the adverse effects of salt stress on both canola cultivars.

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