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Use of fusicoccin in the early selection of durum wheat for tolerance to water stress

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Summary. The use of the fungal metabolite fusicoccin (FC) to induce water stress in durum wheat for the early selection of drought-tolerant genotypes of durum wheat was investigated. The experiments were carried out in a plant-growth chamber using seedlings of two cultivars: Creso and Simeto. Several concentrations of FC (from 10^{-7} to 10^{-5} M) and different modes of application were compared. Changes in the water status of seedlings during the experiments were assessed by measuring variations in fresh weight, leaf water potential, relative water content and transpiration. The results indicated that, under the environmental conditions adopted (20°C, 65% RH, 80 μE m^{-2} s^{-1} continuous illumination), the best simulation of water stress was obtained using cuttings of durum wheat seedlings treated with 10^{-5} M FC solution (6.8 μg ml^{-1}) for at least 24 hours. 'Simeto' tolerated FC-induced water stress better than 'Creso'. This result agrees with previous data from both indoor and outdoor experiments in which other water stress agents were used.

Key words: *Triticum turgidum* var. *durum*, phytotoxin, screening methods, drought resistance.

Introduction

Fusicoccin (FC) is the main phytotoxic metabolite produced by the anamorphic fungus *Phomopsis amygdali* (Delacr.) J.J. Tuset & M.T. Portilla, the causal agent of bud canker or Fusicoccum canker of almond and peach (Graniti, 1962; Ballio *et al*., 1964; Graniti *et al*., 1995a). FC is a diterpene glucoside that acts primarily on the plasma membrane of plant cells, where it stabilises the interaction between the C-terminus of the key enzyme H^+ -ATPase and 14-3-3 proteins, which are the cross-point of a great array of signalling and regulatory pathways (Würtele *et al*., 2003). The association among proteins, FC and H^+-ATPase regulates the activity of the enzyme, priming the proton pump and changing the trans-membrane electric potential. The extrusion of protons and the subsequent acidification of the apoplast induce a number of physiological changes at the cellular, tissue and plant level, which is reflected in cell-wall distension, cell enlargement, promotion of seed germination and callus growth, enhancement of stomatal opening and transpiration, and eventually plant wilting (Sparapano, 1976; Marrè, 1979, 1985; Marrè and Ballarin-Denti, 1985; Clint, 1987; Marrè *et al*., 1989; Graniti *et al*., 1995a; de Boer, 1997; Felle, 1998; Marrè and Albergoni, 1998; Olivari *et al*., 1998; Amtmann *et al*., 1999; Finnie *et al*., 1999;

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Roberts and Bowles, 1999; Camoni *et al*., 2001).

Fusicoccin is a non-selective toxin, being active even in plant species that are not hosts of *P. amygdali*. Its application to plants causes an abnormal opening of the stomata, which do not respond any longer to natural regulating factors, i.e. they remain open even in the absence of light. This results in an increase of transpiration and a corresponding reduction in the water potential (Turner and Graniti, 1969; Graniti and Turner, 1970). The action of FC on the stomata appears to be correlated with an accumulation of K^+ in the guard cells (Turner, 1972; Durbin and Graniti, 1975; Turner and Graniti, 1976) and other biochemical and physiological changes subsequent to the extrusion of H^+. The effects of FC are opposed to those caused by stomatal opening inhibitors, such as abscisic acid (Halloin, 1976; Marrè, 1979, 1985; Pesci and Beffagna, 1984; Behl and Hartung, 1986; Getz *et al*., 1987; Ricciardi and De Giovanni, 1998; Ricciardi, 2001).

Thanks to the non-selectivity of FC for plants and its strong biological activity (it acts at minimum concentrations of 0.1–0.6 mg kg^{-1} f wt), the interest presented by this and related compounds is not limited to plant physiologists and pathologists. FC is used both in basic research (studies of plant physiology in particular) and in applied research (Durbin and Graniti, 1989). One of the most interesting applications consists in substituting the toxin for *P. amygdali* in selective assays of host cultivars and clones, with a view to identifying genotypes poorly sensitive to the toxins of the pathogen (Bottalico, 1976; Graniti *et al*., 1995b). Moreover, since FC and other related metabolites of *P. amygdali* greatly enhance transpiration, they were tested to determine whether they would accelerate: 1. uptake from the soil and translocation in the plant of solutes such as fertilisers, herbicides, systemic fungicides and other chemicals used for plant protection (Amici *et al*., 1980; Marrè *et al*., 1989; Murgia *et al*., 1998); 2. forage drying processes during haymaking (Turner, l970; Bottalico *et al*., 1980); 3. the absorption of CO_2 in environments poorly favourable to gaseous exchange, such as tunnels and greenhouses (Johnson and Rayle, 1976); 4. the response of indicator plants to low concentrations of atmospheric pollutants such as SO_2 (Olszyk and Tingey, 1984); 5. the germination of dormant seeds of various plant species (De Michelis, 1973; Lado *et al*., 1974; Bottalico, 1975; Gul and Weber, 1998; Yoneyama *et al*., 1998).

The potential applications of FC in plant breeding programmes are not limited to the cultivars and clones of natural hosts of *P. amygdali* (some species of Drupaceae), which can thereby be selected for their tolerance or non-sensitivity to the toxin; FC can also be used with other plant species to simulate water stress of various severity, and hence to discriminate drought-tolerant genotypes at an early stage.

Given the serious losses caused by drought to agricultural crops in many parts of the world (Kramer, 1980), this second possibility is potentially important to plant breeders, who can use a few morphological, physiological and biochemical indicators to select cultivars tolerant to water stress (Ricciardi and Steduto, 1988; Fanizza *et al*., 1989; Ricciardi, 1989a, 1989b, 2001; Fanizza and Ricciardi, 1990; Ricciardi *et al*., 1990, 2001).

Materials and methods

The study was carried out on two durum wheat cultivars (*Triticum turgidum* L. var. *durum*, cv. Creso and Simeto) which in previous tests had been found to differ in their reaction to water stress, both natural and simulated by chemical desiccants (sodium salts, polyethylene glycol), cv. Creso being less tolerant than cv. Simeto (Ricciardi *et al*., 1994; Ricciardi and Stelluti, 1995; Ricciardi, 2001).

To determine the range of effective transpiration (Te) in the growth chamber used to perform the experiments, as well as the optimal conditions for FC treatment, six preliminary trials were run on whole or cut seedlings of the two wheat cultivars. The lower part of each seedling was soaked in a test tube (1 cm diam.) containing distilled water (control) or a 10^{-5} M solution of FC, under two temperature and humidity regimes (T=15°C and RH=70%, or T=20°C and UR=65%) and continuous illumination (80 $\mu E\ m^{-2}\ s^{-1}$). After 24 h the relative transpiration in ml mg^{-1} f wt was determined as the ratio of Te to fresh weight of each seedling, applying the formula:

$$Te = (ET - E + \Delta P) / PM$$

where ET is seedling evapotranspiration, expressed as $\mu l\ mg^{-1}$ f wt; E the evaporation of distilled water contained in a test tube in the time unit; DP seedling weight variation in mg (initial f wt - f wt at

the end of the treatment); and PM the seedling mean f wt in mg (arithmetic mean between the initial and final fresh weights).

In the study trials, seedlings of both wheat cultivars, grown in hydroponic culture on Murashige and Skoog (1962) medium till the stage of the second fully expanded leaf, were transferred to a test chamber (T=0°C; RH=65%; continuous illumination = 80 μE m^{-2} s^{-1}). The FC solutions were tested at five concentrations, from 10^{-7} to 10^{-5} M (0.068–6.8 mg l^{-1}) with 0.5 M intervals. Distilled water was used as control. The solutions were either sprayed on the leaves or uniformly applied by a brush or by cotton gauze, or they were absorbed by the seedlings by soaking the lower part of intact (whole) seedlings or seedlings cut at the base of the culm (without roots) in the assay solutions contained in the test tubes.

The effect of the toxin was tested on 10 seedlings per treatment. The weight of each seedling was measured at increasing time intervals (4, 8, 24, 48 and 72 h from the start of treatment) and compared to its initial weight. The leaf water potential (LWP) in bar was also measured using a pressure chamber (Scholander *et al*., 1965). Finally, the relative water content (RWC) in per cent was determined applying the formula:

$$RWC=(PF - PS)/(PT - PS)\times 100$$

where PF is the initial fresh weight of the leaf samples, PT the turgid weight measured after 24 hours from the water soaking of leaves, and PS, the dry weight measured after leaf oven-drying at 70°C for 24 h.

In all cases, the complete randomisation experimental model with three replications was followed. The relatively low number of samples (10 seedlings) per replicate appeared to be adequate both in relation to the uniformity of the traits in the examined cultivars (pure lines) and the low environmental variation due to the experimental conditions of seedling growth, which were always performed in controlled environments. All the data (percent values being transformed to arc sin) were subjected to analysis of variance (ANOVA).

Results

A preliminary set of six experiments was carried out to compare various applications of FC solutions to durum wheat seedlings. The results (data not shown) indicated that leaf wetting by spraying or brushing the solutions in order to produce water deficit or partial wilting was less effective than applying the solutions to the roots or the sectioned culm to be absorbed. It is assumed that in these experiments the toxin applied to the leaves penetrated the wheat leaf epidermis only through the stomatal openings. The Te rate of the wheat seedlings varied according to the environmental conditions. For example, 'Creso' cuttings which absorbed a 10^{-5} M FC solution for 24 h under conditions favouring plant evapotranspiration (20°C and 65% RH), showed a Te of 1.45 μl mg^{-1} f wt, which was 13% more than the Te of 1.28 μl mg^{-1} recorded under conditions of lower evapotranspiration (15°C and 70% RH). The greater Te induced a higher uptake of FC and produced a weight loss of seedlings 34% greater than that shown by seedlings under the less favourable environmental conditions.

In the main set of experiments, the seedlings either with or without roots were soaked in the FC solutions in a test tube. The mean absorption was 1 μl solution per mg of f wt on the first and second day, but in the following 24 h it became progressively lower. To make the tests less time-consuming, instead of monitoring Te, percent variation in seedling weight was determined five times during the three-days of the experiments.

Analysis of variance (ANOVA) of the values obtained is shown in Table 1. The two cultivars tested (G) reacted similarly (difference not significant) to the treatments, whether applied to the whole seedlings or to the cuttings. However, the variation in weight of both seedlings and cuttings was affected by the concentration of the FC solutions (C) and by the duration of the treatment (P). The statistical significance of the interactions between cultivars and concentrations of FC (G×C), and (for cuttings only) between cultivars and duration of treatment (G×P), indicated that the behaviour of the cultivars differed.

During the first 8 hours of treatment, the change in weight of the seedlings was relatively moderate, irrespective of the FC concentration and the cultivar (Fig. 1). The treatment effect became significant after 24 h, especially for the cv. Creso. The most effective FC concentration was 10^{-5} M. The

Table 1. ANOVA on the percent variation in weight (compared with controls) of seedlings of two cultivars of durum wheat during the experiments (4, 8, 24, 48 and 72 h). FC solutions were assayed at 5 concentrations (10^{-7}–10^{-5} M) on whole and cut seedlings. Control seedlings received distilled water.

Source of variation	Degrees of freedom	Seedlings[a]	
		Whole	Cut
Cultivar (G)	1	n.s.	n.s.
FC concentration (C)	5	***	***
Treatment (P)	4	***	***
G × C	5	***	*
G × P	4	n.s.	**

[a] n.s., not significantly different; *, **, ***, significantly different at P=0.05, 0.01 and 0.001, respectively.

Table 2. Leaf water potential and relative water content of cut seedlings of two cultivars of durum wheat during absorption of a 6.8 mg ml^{-1} (10^{-5} M) FC solution or distilled water (control) under a controlled environment[a].

Cultivar	Treatment period (hours)							
	4		8		24		48	
	FC	Control	FC	Control	FC	Control	FC	Control
	Leaf water potential (-bar)							
Simeto	10.7	10.8	10	11.7	14.9**	11.5	21.7	17
Creso	11.2	10.7	9.7	11	13.6**	10.7	**28.3****	16.3
	Relative water content (%)							
Simeto	93.2	93.4	89.9	96	72.3**	95.5	60.3	85.8
Creso	92.2	93.8	89	95	**68.9****	93.7	53.9**	88

[a] A plant-growth chamber at 20°C, 65% RH and continuous illumination (80 μE m^{-2} s^{-1}). Values are the mean of 3 replicates, 10 seedlings per treatment. For each treatment period, two asterisks (**) indicate a significant difference (P=0.01) between FC-treated and control wheat cuttings. Values in boldface indicate significant differences (P=0.01) between cultivars.

most serious water loss at this concentration was in the cut seedlings of 'Creso', with a reduction in weight (P=0.05) of 13% after 24 h, 25% after 48 h and 32% after 72 h. The effect of the same FC concentration on whole seedlings of 'Creso' was lower and slower (10% loss of weight after 48 h). At the lowest FC concentration, the increase in weight was greater in the whole seedlings that in the cut ones, probably because absorption of the solution through the roots was slow, and because low doses of FC had an auxin-like effect on the cell wall, stretching it and increasing subsequent water uptake by the plant tissues.

Whole seedlings of cv. Simeto did not show severe water stress at any FC concentration; cut seedlings however showed a progressive but modest loss of weight (12% at the end of the trial; P=0.05%) at the most concentrated FC solution (10^{-5} M). 'Simeto' was more tolerant than 'Creso' to the stress caused by the toxin.

As expected, the LWP and the RWC were changed by FC treatment of the wheat seedlings. Analysis of variance of the LWP after treatment with FC at 10^{-5} M for 48 h detected that all the sources of variation and their interactions were significantly different (P=0.05 between cultivars; P=0.01 between the other parameters). The RWC differed significantly only with the length of the treatment, the treatment itself (as compared to the controls) and their interaction.

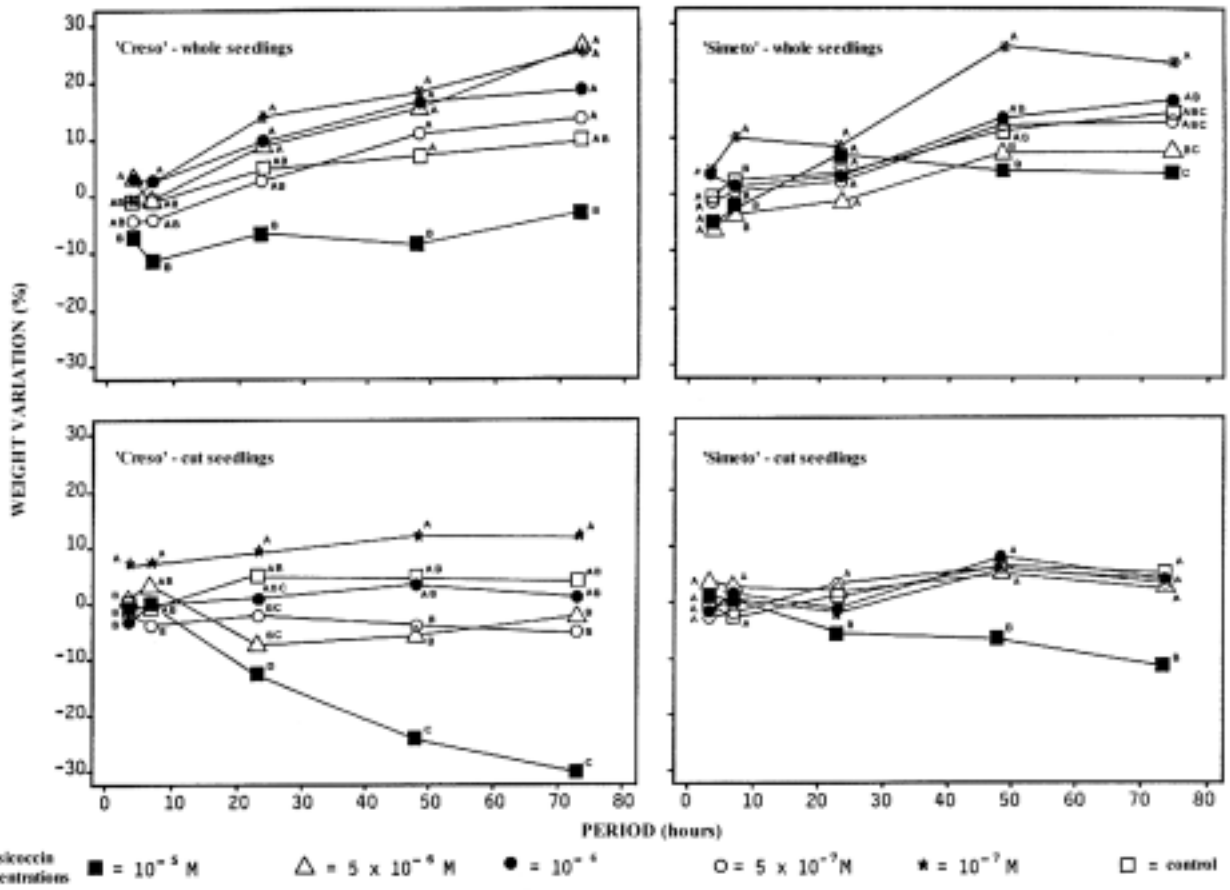

Fig. 1. Per cent variation in weight of whole or cut seedlings of two durum wheat cultivars during the absorption of FC solutions at various concentrations. Values are the means of 5 replications. For each treatment period, values without any common letter differ from each other at P=0.05 (Duncan's test).

The mean LWP and RWC values, recorded four times during the first 48 h of the experiment are shown in Table 2. In general, as the FC treatment went on, the LWP increased (negative values higher) and the RWC went down. RWC values of both cultivars were significantly different from the controls after 8 h, whereas LWP values did not become significant until after 24 h. The two wheat cultivars further differed significantly in that 'Simeto' after 24 h had a higher RWC and, after 48 h, a lower LWP than 'Creso', signifying that 'Simeto' had a better tolerance to FC-induced water stress.

Discussion

To improve the effectiveness of plant breeding programmes for water stress tolerance, some morphological, biochemical and physiological features, preferably tested by early assays, may be added to the usual selective criteria commonly based on the genotype productivity under stress. Emphasis is also laid on finding and developing stress-simulation methods which, like the assays mentioned above, are not only practicable on sufficiently sensitive seedlings, but are also reproducible without much difficulty. Compared with traditional methods, which are mostly conducted in the field and in areas where water deficits are not regularly distributed throughout the seasons of the year, these stress-simulation methods will make it possible to produce a greater number of generations or selective cycles per year when breeding durum wheat

for drought tolerance. They will also reduce the experimental errors inherent in this type of trial, especially under field conditions. These errors often mask differences in the responses of plants to stress, and hence differences between genotypes.

Although only two cultivars among the most commonly grown in southern Italy and reacting differently to drought were tested, the results suggest that FC can be used to simulate water-stress in rapid assays. They also suggest that, in experiments on other wheat genotypes, FC could be used successfully alongside other methods of screening durum wheat for drought tolerance. FC can be used in the early selection of durum wheat genotypes tolerant to drought.

The absorption of the solutions through the cut culm was the most effective of the ways to apply FC solutions to wheat seedlings and bring about a relatively quick loss of leaf tissue turgor.

To achieve its effect on durum wheat, the FC concentration must be higher (at least 6.8 $\mu g\ ml^{-1}$) than the FC concentrations of 0.07 to 0.7 $\mu g\ ml^{-1}$ required to cause similar or even more severe effects on more sensitive and more actively transpiring herbaceous species (tomato, for instance: Graniti, 1962; Bottalico, 1971), and an absorption time of at least 24 h is needed.

Notwithstanding the "hardness" of durum wheat leaves, the absorption of FC solutions through the roots or the xylem – the latter being more effective than the former – caused physiological changes in the known effects of FC on plant cells, particularly stomatal guard cells. These changes produced immediate increases in transpiration and water uptake, with a consequent gain in the weight of treated seedlings. Subsequently, at the most effective FC concentrations, the impairment of the permeability of plant cell membranes dramatically decreased both the water potential and the cell turgor of the leaves, with a progressive reduction in seedling weight. The absorption of a 10^{-5} M solution of FC for 24 h by cuttings of 'Creso' increased transpiration by 50% over the controls and decreased cutting weight by 10%. Water stress and weight loss became more marked throughout the experiment, and eventually leaves began to show wilt symptoms in the form of rolling and twisting of the lamina along the main leaf axis.

In conclusion, the effect of FC on the water status of two cultivars of durum wheat demonstrated that this toxin simulates drought stress in the short term. The genotype response to stress caused by FC is in general agreement with previous studies both in artificial environments and in the field (Blum, 1988; Ricciardi *et al*., 1994; Ricciardi and De Giovanni, 1998; Ricciardi, 2001): the wheat cv. Simeto has a greater tolerance to water stress than 'Creso'. FC thus seems to be a useful tool to simulate water stress not only in herbaceous and broadleafed plants, but also in sclerophylls, grasses, and cereals such as durum wheat.

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