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ABA level, proline and phenolic concentration, and PAL activity induced during cold acclimation in androgenic Festulolium forms with contrasting resistance to frost and pink snow mould (*Microdochium nivale*)

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ABSTRACT

The metabolic changes in foliar abscisic acid, proline and phenolic concentration, and PAL activity induced during cold acclimation in androgenic forms of Festulolium contrasting in terms of their resistance to frost and *Microdochium nivale* were studied. The frost-resistant and snow mould-resistant (cross-tolerant) genotype (561) was characterized by a higher ABA amount and higher PAL activity compared to those of the frost and snow mould-susceptible genotype (621) during cold acclimation. The obtained results suggest that both these parameters are involved in a cross-tolerance mechanism relating to frost and snow mould resistance.

1. Introduction

Winter-hardiness of grasses and cereals depends mainly on frost tolerance and resistance to snow mould, which may be caused by many different fungus species. In Poland, snow mould is caused mostly by *Microdochium nivale* (Fr) Samuels & Hallett [1]. *M. nivale* evokes injuries, which can lead ultimately to plant death observed soon after snow melting [2,3]. A wide diversity of grass and cereal resistance to snow mould was demonstrated [1]. The lack of resistance to both frost and snow mould fungi is the main reason for the breeding limitations of new cultivars of winter grasses and cereals. The susceptibility of those species to snow mould is also the main cause of significant yield losses.

Plants exposed to one stress factor can increase resistance to another and that mechanism is called "cross-tolerance" [4–6]. Cross-tolerance exists also between biotic and abiotic stress. Low temperature increases plant susceptibility to fungi, and conversely, winter pathogen infection decreases plant resistance to frost [2].

Cold acclimation of plants develops in two stages: during pre-hardening, when the day temperature ranges 12–16 °C and at non-freezing night temperature and during hardening at temperatures 2–5 °C. During pre-hardening and hardening, structural and biochemical changes adapting plants to winter conditions are observed. These changes relate to the reorganization of plasma membranes, synthesis of dehydrins, protection of membranes from frost damage, production of cryoprotectants preventing ice formation outside the cells, strong dehydration of cytoplasm and a reduction of the water potential of cells [7]. The acclimation processes should therefore improve plant resistance both to frost and winter pathogens [2].

During cold acclimation, an important role is played by abscisic acid (ABA), which regulates water balance and activates antioxidant enzymes, but which may inhibit synthesis of β-1,3-glucanases [8–10]. ABA synthesized during cold acclimation in forage and a grain grass is mainly involved in the response to abiotic stresses, increasing plant resistance. In the case of biotic stress, ABA mostly reduces plant resistance. However, some research shows that abscisic acid can also enhance resistance to pathogens through its positive effect on callose deposition [11,12].

Accumulation of proline occurs in response to many abiotic stresses including drought, salinity and frost as well as biotic stresses such as pathogen infection [13–15]. However the mechanism of proline enhancing stress resistance is still debatable. The main role of proline is its involvement in osmoregulation, and the

Abbreviations: ABA, abscisic acid; ELISA, enzyme-linked immunosorbent assay; PAL, phenylalanine ammonia-lyase; PPFD, photosynthetic photon flux density [μmol m^{-2} s^{-1}]; RT, relative turgidity; SE, standard error; Ψ_o, osmotic potential.

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stabilizing of sub-cellular structures and scavenging of free radicals. ABA is required for proline accumulation in stressed plants. Verslues and Bray [16] observed that ABA applied to unstressed plants, elicited much lower levels of proline accumulation in comparison with plants treated with a stress factor. According to these authors, it could be caused by an ABA-independent effect of stress on proline level or a stress-induced change in response to ABA.

The mechanisms of frost and pathogen resistance include biochemical changes of phenolic accumulation and phenylalanine ammonia-lyase (PAL) activation. The phenylpropanoid pathway is activated under the influence of different stress factors [17]. Phenolic synthesis is recognized as a result of signalling processes initiated very quickly after injury, an attack of pathogens or elicitation [18]. An increase in PAL activity could be a marker of plant reaction to environmental stress [19]. PAL is the key enzyme of the phenylpropanoid pathway leading to the synthesis of many derivatives of hydroxycinnamic acid and alcohols. Cold acclimation resulted in a pronounced increase in PAL activity in rape plants, which was associated with a marked accumulation of phenylpropanoid compounds in leaves [20,21].

The aim of the present study was to quantify the pre-hardening and cold acclimation effect on abscisic acid level, proline and phenolic concentration, and PAL activity in the leaves of Festulolium. The aim of the experiment was to investigate whether the metabolic changes in all the aforementioned parameters, induced during cold acclimation of androgenic forms of Festulolium, are related to their resistance to frost and *M. nivale*. The experiment was also conducted to check whether the measured parameters can be used as a physiological marker in the evaluation of the degree of snow mould resistance in plants of the studied species.

2. Material and methods

2.1. Plant material

The study was performed on two genotypes nos. 561 and 621, derived from the Polish Festulolium cultivar 'Rakopan' [22]. On the basis of field observations, genotype 561 was recognized as the more resistant to frost and *M. nivale* as genotype 621 (data not shown). The 2-year-old plants of both these genotypes were transferred from field to a greenhouse, divided into cuttings and grown at a constant temperature of 18 °C. After six weeks, the plants were pre-hardened for two weeks at 12 °C under a 10 h photoperiod with a light intensity of 250 μmol m^{-2} s^{-1} PPFD. Then plants were hardened for three weeks at 2 °C under an 8 h photoperiod with a light intensity of 150 μmol m^{-2} s^{-1} PPFD. The experiment was performed every year for three years. Each year the resistance to frost and pathogen was tested. During the last experiment measurements were conducted to check whether some biochemical parameters can be used in the evaluation of the degree of snow mould resistance.

Analyses of ABA concentration, osmotic potential (Ψ_o) and relative turgidity (RT) were completed during the first hours of cold acclimation i.e. after 6, 12, 30 and 54 h of cold treatment (2 °C). Total phenolic content and PAL activity were measured after 2 weeks pre-hardening at 12 °C and 3 weeks cold acclimation at 2 °C.

2.2. Artificial inoculation

After cold acclimation artificial inoculation with *M. nivale* mycelium was completed. The *M. nivale* strain 2/01 was isolated from *Lolium perenne* in the Plant Breeding and Acclimatization Institute in Radzików, Poland by Prof. Maria Prończuk. The inoculum was prepared by growing the fungus in a soil medium [1] at 18–20 °C for 7 days in darkness. After colonization by the mycelium the soil medium was macerated. Inoculation was made by adding 1 g of the inoculum per plant. The inoculated and non-inoculated (control) plants were covered with moistened blotting paper and black foil to retain a high humidity. Next all plants were incubated for 5 weeks at 1 °C in darkness. At the end of that period the paper was removed and plants were grown for 10 days at 12 °C (100 μmol m^{-2} s^{-1} PPFD). Directly after paper removal the plant resistance to snow mould was scored.

2.3. Evaluation of snow mould resistance

Evaluation of the plants response to disease was conducted using the visual rating system (0–5) described by Hartman et al. [23] with a slight modification where: 0 means a plant without symptoms and 5 means a whole plant is infected. An Average Severity Index (ASI) was calculated from these ratings according to the formula:

$[(n \times 0) + (n \times 1) + \ldots\ldots + (n \times 5)]N^{-1}$; n = number of plants corresponding to each disease rating (0–5), N – total observations.

ASI was calculated in 5 replications as an average from five pots (9 plants in pot, each pot = one replicate). Decreased values of ASI mean an increase in plant resistance to a pathogen.

2.4. Evaluation of freezing tolerance

Hardened plants were frozen as described by Rapacz et al. [24] at −8, −11, −14 °C for 2 h, after which the plants were transferred to a greenhouse at 12 °C for 10 days. After this time, the evaluation of freezing damage was done using the visual 0–9 rating system according to Larsen [25], where 0 means plants without green leaves, while 9 means plants with only green leaves,. The evaluation was completed for 5 pots each containing 9 plants (one pot = one replicate).

2.5. ABA concentration

Plant material was freeze-dried and ground in a ball mill (Retsch, Kroll, Germany). Cold distilled water was added. The samples were heated for 1 min in boiling water and then shaken overnight at 4 °C. The next day, the extracts were centrifuged for twenty minutes in a refrigerated centrifuge at 18,000*g* (MPW-350R, Poland). ABA was measured in the supernatant using an indirect enzyme-linked immunosorbent assay (ELISA) in accordance with the protocol described by Walker-Simmons and Abrams [26]. The antibody used was MAC 252 (Babraham Technix, Cambridge, UK). The measurements were made for five samples collected from different plants of each treatment.

2.6. Osmotic potential (Ψ_o)

The osmotic potential of leaf cells was measured with a dewpoint microvoltometer (HR 33T, Vescor) supplied with a C-52 Sample Chamber. Cell sap was extracted with a fixed force from a leaf fragment (7 mm in diameter) collected from the youngest fully expanded leaf and frozen in liquid nitrogen. Each assay was performed in ten replications representing ten leaves from different plants for each treatment.

2.7. Relative turgidity (RT)

Directly after 6, 12, 36, 54 h of growth at 2 °C the relative turgidity (RT) of leaves was determined. Immediately after sample collecting their fresh weight (FW) was determined and next the samples were immersed in distilled water in a closed container (100% RH) for 24 h. After that time the full turgor weight (FTW) was determined. Dry weight (DW) was the weight after 24 h of drying at 85 °C. The relative turgidity was calculated from the formula