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Factors contributing to enhanced pink snow mould resistance of winter rye (*Secale cereale* L.) – Pivotal role of crowns

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ABSTRACT

Frost tolerance, resistance to *Microdochium nivale* and associated biochemical changes were investigated in leaves and crowns of six winter rye inbred lines. Investigated lines differed in their susceptibility to snow mould but not in frost tolerance. It was shown that winter rye resistance is linked to defence response in crowns expressed by higher activity of antioxidant enzymes – CAT, SOD and total peroxidases – on the 1st day after inoculation and β-D-glucosidase activation during the course of pathogenesis. It could be postulated that changes in leaves are attributed to reaction to cold while changes in crowns are attributed to pathogen reaction.

1. Introduction

Microdochium nivale (Fr) Samuels & Hallet is a common pathogen of many plant species, including wheat, rye, barley, oats, turf and forage grasses, in cold and moderate regions of the northern hemisphere [1]. Thus, the psychrophilic fungus is distributed over a wide range of temperatures, but its development is favoured by high humidity and sub-zero temperatures. The basic frost tolerance mechanism of *M. nivale* is based on the constitutive abundance of polyunsaturated fatty acids among the phospholipids in cell membranes [2]. The main sources of *M. nivale* infection are resting spores and mycelium in the soil or on the plant debris [3]. The fungus attacks different tissues, starting from the endoderm and cortex of the root crown and then spreading to the epidermis of the leaves and leaf sheaths [4]. Infection with *M. nivale* consists in the spread of the pathogen and cell wall penetration. It begins in the fall, before or after snowfall [5]. After the snow melts, a pinkish-white fungal mycelium appears on the leaves, and in the course of time, it turns a characteristic salmon-pink colour. Leaves and leaf sheaths turn a light to dark brown colour.

One of the most important mechanisms of plant resistance to pathogens is hypersensitivity response (HR), which includes the generation of ROS and local cell death [6], but the response depends on the pathogen's lifestyle [7]. Pathogen recognition is followed by an oxidative burst in the apoplast and an increase in peroxidases activity [8]. Apoplastic and cell wall-bound peroxidases take part in lignin and suberin formation [9]. They also catalyse hydrogen peroxide (H_2O_2) reduction taking electrons from various donor molecules such as phenolic compounds.

Enzymes such as superoxide dismutases (SOD, EC. 1.15.1.1), peroxidases (class III peroxidases, POD, EC.1.11.1.7) and catalases (CAT, EC 1.11.1.6) are responsible for ROS homeostasis in different compartments of plant cell [10]. Superoxide dismutase is the first protective enzyme detoxifying ROS by dismutation of superoxide radical to hydrogen peroxide and oxygen. In turn, in peroxisomes catalase decomposes hydrogen peroxide to water and oxygen. Hydrogen peroxide and superoxide radical ($O_2^{\cdot-}$) are the major ROS responsible for the oxidative burst. This phenomenon consists in a rapid and transient release of high levels of ROS in plant cells in response to external stresses, including activity of pathogens such as fungi.

ROS production and neutralisation differ in response to biotrophs and necrotrophs. ROS generation and low antioxidant activity are more favourable for necrotrophic pathogens, while biotrophic pathogens induce activation of antioxidant systems. For necrotrophs, disintegrated plant tissue is a source of nutrients for pathogen development, while biotrophs favour living cells [7].

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Among the most important elements of defence response to fungal infection are isoflavones, secondary metabolites which may occur in a free state or as esters of glycosides [11]. From glycosides free isoflavone aglycones may be released by specific glucosidases such as β-D-glucosidase (EC 3.2.1.21). This enzyme also takes part in cell wall reorganisation by releasing glycosidase-bound phenolics [12]. An increase of β-D-glucosidase activity was observed by many authors in plant-fungal pathogen interaction; for example in the case of grapes, apple fruits, and lettuce leaves infected by *Botrytis cinerea* [13,14], as well as pea attacked by *Fusarium solani* [15].

Winter rye is a cereal frequently cultivated in Poland, Germany and Russia, because it is well adapted to the weather conditions of these countries. Compared to other cereals, rye is the most winter-hardy cereal grain which can germinate and grow at low temperatures, but it is also known to have low snow mould resistance. Although fungicidal treatment is, for now, the most effective means of control of *M. nivale* development, there are several drawbacks associated with its use, predominantly the risk of resistance development and environmental hazards. An alternative to chemical methods of disease control with pesticides is to breed plants resistant to pink snow mould. The knowledge of defence response patterns of winter rye cultivars in order to establish possible defence mechanisms, apart from purely scientific significance, would certainly be of great importance for winter rye breeders, who would be provided with additional selection markers.

This study suggests that the interaction between pathogen and plant depends on the ways in which the pathogen penetrates different tissues, and can be correlated with the metabolic pathways of some antioxidant enzymes in different plant organs. Therefore, the focus was on differences or similarities in interactions between leaves and crowns and the pathogen. One of the aims of the study was to identify the reason for low pink snow mould resistance in winter rye and in particular, to establish the patterns of antioxidants activity and plant β-D-glucosidase activity, typical for lines resistant to the disease.

2. Material and methods

2.1. Plant material

The experiment was carried out using three-week-old seedlings of six inbred lines of winter rye (nos. 1, 2, 4, 5, 22, 23). The seeds were sown in pots [20 plants per φ 25 cm pot) containing a mixture of soil: peat: sand (2: 2: 1 v/v/v) at pH 5.8 and cultured for three weeks in a greenhouse at 18 °C (day/night) in daylight (the experiment was conducted in October, latitude: 50°03′ north; longitude: 19°55′ east). The plants were supplemented with light: 250 μmol m^{-2} s^{-1} PPFD (AGRO Philips sodium lamps) up to a 12 h photoperiod and were fertilised weekly with Hoagland's liquid medium. Next, the plants were pre-hardened in a growth chamber for two weeks at 12 °C under a 10 h photoperiod with a light intensity of 250 μmol m^{-2} s^{-1} PPFD and cold acclimated for three weeks at 2 °C under an 8 h photoperiod with a light intensity of 150 μmol m^{-2} s^{-1} PPFD.

2.2. Frost tolerance

Frost tolerance was estimated in an independent experiment after 3 weeks of cold acclimation according to the method described by Rapacz et al. [16]. Cold acclimated plants were frozen at −9, −12, −15 °C, next the evaluation of freezing damages was performed, using the visual rating system 0–9, where 0 means plant without green leaves and 9 means plant with green leaves only. The temperature, causing a 50% depression of regrowth – RT_{50} was estimated from linear regression fitted to the sigmoid relationship between the freezing and regrowth score.

2.3. Artificial inoculation with M. nivale

After the cold acclimation at the 3-leaves stage, plants were inoculated with the *M. nivale* mycelium. The *M. nivale* strain was isolated from *Secale cereale* seeds by Prof. Maria Prończuk from the Plant Breeding and Acclimatization Institute in Radzików (Poland). The inoculum was prepared by growing the fungus in a soil medium [17] at 18–20 °C in darkness for 7 days. After colonization by the mycelium, the soil medium was macerated. Inoculation was made by adding 1g of the inoculum per plant to the soil. The inoculated and non-inoculated (control) plants were covered with moistened blotting paper and plastic foil to keep a high humidity. Next, all the plants were incubated for 5 weeks at 1 °C in darkness. At the end of that period, the blotting paper and foil were removed, and the plants were grown for 10 days at 12 °C in a light of 100 μmol m^{-2} s^{-1} PPFD.

On the 1st, 7th and 13th day after inoculation, catalase (CAT), superoxide dismutase (SOD), non-specific peroxidase (PX) and on the 1st and 13th day, β-glucosidase activity were determined. Samples were collected from the middle part of fully expanded youngest leaves and whole root crown tissues.

2.4. Snow mould resistance

2.4.1. ARI evaluation

Snow mould resistance was estimated on the basis of the regrowth after *M. nivale* inoculation. At the end of the 5 weeks of incubation at 2 °C in darkness, the blotting paper and foil were removed and the leaves were then cut and allowed to recover (3 days at 12 °C in 100 μmol m^{-2} s^{-1} PPFD light and the next 7 days at 18 °C in 250 μmol m^{-2} s^{-1} PPFD light).

Plant regrowth was evaluated as described by Płażek et al. [18] using the visual rating system (0–5), where '0' means a plant without any visible symptoms of infection and '5' means a completely dead plant with no signs of leaf elongation. The Average Regrowth Index (ARI) was calculated from these ratings according to the formula: $[(n \times 0) + (n \times 1) + \ldots\ldots + (n \times 5)] \cdot N^{-1}$; n – number of plants corresponding to each disease rating (0–5), N – total observations. ARI was calculated in five replicates as an average from five pots (25 plants in a pot, each pot = one replicate). ARI evaluation was done on the basis of two independent experiments.

2.4.2. Evaluation of dry weight of recovered leaves

Plant regrowth was also evaluated through an estimation of the dry weight of recovered leaves. The living tissue of inoculated and non-inoculated plants was cut above the soil level and dried at 60° for 3 days. Relative regrowth was calculated as follows: dry weight of inoculated plants divided by dry weight of non-inoculated control plants and expressed as percentage values.

2.5. Assay of superoxide dismutase (SOD) (E.C.1.15.1.1) activity

Superoxide dismutase activity was estimated according to Droillard et al. [19]. Leaf samples were homogenised at 4 °C with a 50 mM phosphate buffer (pH 7.8) and 1% PVPP and centrifuged at 16,000 × g. The reaction mixture contained 50 mM potassium phosphate (pH 7.8), 1 mm EDTA, 1 unit of catalase, 56 mM nitroblue-tetrazolium (NBT), 0.1 mM xanthine, 0.03 units of xanthine oxidase and extract from the tissue. The absorbance was monitored at 560 nm. The inhibition percentage of NBT reduction is a measure of SOD activity. One unit of SOD is the amount of extract that provides half-maximum inhibition. The determination of SOD activity was done in five replicates (five independent samples collected from different plants).