Pesticide Biochemistry and Physiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

The influence of chosen fungicides on the activity of aminopeptidases in winter oilseed rape during pods development

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ARTICLE INFO

Keywords:
Oilseed rape
Fungicides
Aminopeptidases
Crop yield

ABSTRACT

Cultivation of oilseed rape requires application of specific fungicides. Besides their protective role, they can potentially influence the expression and activity of crucial enzymes in the plant. Among the large number of enzymes expressed in plants, aminopeptidases play a key role in all crucial physiological processes during the whole life cycle (e.g. storage protein mobilization and thus supplying plant with needed amino acids, as well as plant aging, protection and defense responses). In the present paper, we evaluate for the first time, the influence of the treatment of winter oilseed rape with commercially available fungicides (Pictor 400 SC, Propulse 250 SE and Symetra 325 SC), on the activity of aminopeptidases expressed in each plant organ (flowers, leaves, stems and pods separately). Fungicides were applied once, at one of the three stages of oilseed rape development (BBCH 59–61, BBCH 63–65 and BBCH 67–69). The aminopeptidase activity was determined using six different amino acid *p*-nitroanilides as substrates. The results have shown, that in control plants, at the beginning of intensive pods development and seeds production, hydrophobic amino acids with bulky side chains (Phe, Leu) were preferentially hydrolysed. In control plants, the activity was ~3.5 times higher in stems and pods, compared to leaves. The treatment with all pesticides caused significant increase in aminopeptidases hydrolytic activity toward small amino acids Gly, Ala as well as proline, mostly in flowers and leaves. These amino acids are proven to be crucial in the mechanisms of delaying of plant aging, development of better resistance to stress and plant defense. It can be suggested, that studied fungicides enhance such mechanisms, by activating the expression of genes coding for aminopeptidases, which are active in hydrolysis of *N*-terminal amino acids such as Gly, Ala, Pro from storage peptides and proteins. Depending on fungicide, the major increase of aminopeptidase activity was observed after application at BBCH 67–69 (Pictor 400 SC and Symetra 325 SC) and BBCH 63–65 (Propulse 250 SE) stages of development. Our study revealed, that agrochemical treatment and time of application, influenced the expression and activity of aminopeptidases, even though they were not molecular targets of applied fungicides. Since aminopeptidases are widely distributed throughout all organisms and are crucial in many key physiological processes, it can be expected, that factors influencing their expression and activity in plants, can also influence these enzymes in other organisms, especially humans and other mammals.

1. Introduction

Rapeseed oil production is very important not only because of its application in food industry, but also in petrochemical branch. Additionally, post-extraction meal and bagasse from oilseed rape are important sources of proteins in animal feedstuffs production. Oilseed rape (*Brassica napus*) is known in Europe since XVII century [1]. Due to the climatic conditions, it is the most important oilseed crop in Europe. One of the most dangerous threats for oilseed rape development and crop yield are plant pathogenic fungi. *Sclerotinia sclerotiorum* is a plant pathogenic fungus, that can cause a disease called white mold, and infects hundreds of plant species worldwide. Diseases caused by *Sclerotinia* are serious problem for many plants [2], including oilseed rape cultivation, since the infection can occur on leaves, stems and pods at different stages of development [3]. Another disease that has a great impact on the oilseed rape growth is *Alternaria* disease caused by *Alternaria brassicae, A. brassicola* or *A. alternata*. In oilseed rape *A. brassicae* is the dominant invasive species. *Alternaria* pathogens usually

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https://doi.org/10.1016/j.pestbp.2018.04.008
Received 14 June 2017; Received in revised form 5 March 2018; Accepted 23 April 2018

cause damping-off of seedlings. Seed infection reduces germination and seedlings vigour, and affects the sale and use of infected/infested seeds [4]. Many different types of fungicides have been applied to effectively overcome fungal infections. Among them triazoles play important role. They inhibit cytochrome P-450 14 alpha-lanosterol demethylase and thus block the ergosterol biosynthesis in fungal cells [5–8]. Recently, the special attention has been paid to prothioconazole, which has a broader spectrum of activities. Additionally, it is also an effective preventive agent [9]. Another group of fungicides - strobilurins interfere with energy production by inhibiting mitochondrial electron transport chain between cytochrome b and cytochrome c1 at the Qo (quinol oxidation) site and thus prevent generation of ATP, which breaks the energy cycle of the pathogen [10,11]. Studies have also shown that strobilurins inhibit plant respiration [12], and cause other physiological changes, which affect production of phytohormones and delay leaf aging. Preparations from this group reduce stomatal conductance and water consumption, which result in increased photosynthetic activity, they also increase the activity of antioxidant enzymes [13–15].

The new trends in plant protection against fungal diseases is set by new active components (penthiopyrad, fluopyram, penflufen, sedaxane, bixafen, isopyrazam and fluxapyroxad) belonging to SDHI group of fungicides (succinate dehydrogenase inhibitors). They block fungal respiratory chain at complex II (inner mitochondrial membrane), thus cutting off the energy supply, and limiting availability of building material for the synthesis of important components, necessary to build cells of pathogens. They are highly effective at rather low rates, do not exhibit cross resistance with other classes of fungicides and have broad spectrum of activity [16–20].

The application of fungicides can influence not only the molecular targets but also other crucial enzymes in the plant, causing some unexpected cultivation problems. Plant proteases, including aminopeptidases (APs; EC 3.4.11.XX), play significant role in many life processes such as storage protein mobilization during germination [21], maturation and degradation. Aminopeptidases, enzymes responsible for the cleavage of *N*-terminal amino acids from proteins and peptides, are widely distributed throughout all organisms (bacteria, plants, animals, humans), and are crucial in many key physiological processes. The comparison of amino acid sequences revealed, that aminopeptidases having similar function in different organisms show homologies, especially in catalytically important residues. It can be expected, that factors having impact on expression and activity of aminopeptidases in plants can also influence these enzymes in other organisms, especially humans and other mammals.

Elevated aminopeptidase activities were often detected in organs of plant with accelerated protein turnover, such as leaves and petals during senescence, fruits maturation or in germinating seeds [22]. Moreover, it was proven that these enzymes take part in defense responses [23–25]. Part of the published research was focused on aminopeptidase activity in plants subjected to various biotic and abiotic factors, during germination and growth processes. In pigeon pea (*Cajanas cajan*) plants, infected with *Fusarium oxysporum* and *Aspergillus niger* var. *niger,* changes in aminopeptidase activity were observed during 72 h of germination. Two aminopeptidases were identified (AP1 and AP2). AP1 activity was significantly induced by infection with fungi [23]. The most explored and studied enzyme - acidic leucine aminopeptidase (LAP-A) from tomato – was described as being involved in response to different factors, such as water deficiency, salinity stress, plant hormones (abscisic acid or systemin), wounding, heavy metals (e.g. cadmium) or even attack of herbivores (e.g. insects) [25–27]. Leucine aminopeptidase 2 was proven to be a salt-responsive biomarker in leaves of diploid wheat *T. monococcum* [28]. Changes (mostly increase) in aminopeptidases expression and activity in response to harmful factors were described for many plants, e.g. bean *Phaseolus vulgaris* [29], triticale barley [30,31], pigeon pea [23,24], black nightshade (*Solanum nigrum*) [32].

There are only few data about APs from *Brassica napus*. Recently [33], aminopeptidases from non-germinated seeds of winter oilseed rape (*Brassica napus*) were characterized. To the best of our knowledge, there are no reports discussing the influence of plant protection agents on the expression and activity of plant aminopeptidases. Since aminopeptidases play crucial role not only in plants but in all living organisms, this study can show the possible impact of such agents on human and other mammals health.

Here we present for the first time, the influence of selected fungicides, applied at three different timings, on the expression/activity of aminopeptidases in winter oilseed rape. The activity was determined in different plant organs, namely flowers, leaves, stems and pods. One of the fungicides – Pictor 400 SC contains strobilurin (dimoxystrobin) as an active ingredient, and boscalid which is succinate dehydrogenase inhibitor (SDHI). In Symetra 325 SC, SDHI - isopyrazam serves as an active ingredient, and azoxystrobin as a respiration inhibitor (strobilurin). Propulse 250 SE is two-component fungicide containing SDHI (fluopyram) and sterol biosynthesis inhibitor (prothioconazole). The goal of the paper was to show that studied fungicides, besides their major role, could also be involved in defense and protection mechanisms in winter oilseed rape, by enhancing the expression/activity of specific aminopeptidases. These aminopeptidases catalyse the hydrolysis of storage proteins, especially cleavage of *N*-terminal amino acids, crucial in the defense and protection processes. The study was performed using partially purified protein extracts from different winter oilseed rape organs. The relationship between applied fungicides, aminopeptidases activity and oilseed rape crop yield as well as thousand kernels weight was also discussed.

2. Experimental

2.1. Materials and reagents

L-leucine *p*-nitroanilide (Leu-*p*NA), L-alanine *p*-nitroanilide (Ala-*p*NA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Bovine serum albumin fraction V was obtained from Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Acros Organics (Geel, Belgium). Dimethyl sulfoxide (DMSO), ammonium sulfate, sodium chloride and Triton X-100 were from POCH (Gliwice, Poland). Glycine *p*-nitroanilide (Gly-*p*NA), L-methionine-*p*-nitroanilide (Met-*p*NA), L-phenylalanine-*p*-nitroanilide (Phe-*p*NA), L-proline-*p*-nitroanilide (Pro-*p*NA) were synthesized according to the known procedures [34,35]. All other chemicals were of high analytical grade and used as purchased. Winter oilseed rape cv. Hybrirock F1 was obtained from Institute of Plant Protection National Research Institute, Department Sosnicowice, Poland. Tested fungicides are listed in Table 1.

2.2. Equipment

The following equipment was used in the experiments presented in the paper: Spectrophotometer UV/VIS Jasco V-650, centrifuge 5804R Eppendorf, vortex PV-1 from Grant-bio, homogenizer ErgoMix Bosch, Direct Detect™ Quantitation System from Merck Millipore.

2.3. Plant cultivation and fungicide treatment

During the 2014/2015 growing season in Slupsk, Province of Upper Silesia, researchers conducted controlled plot trials in randomized block design, set up with four repetitions on a flat ground. The trials were carried out in brown earth soil of good wheat complex. The soil texture was loamy sand with pH 6.5, the content of organic matter was 1.9%. Mineral fertilization complied with the requirements for winter oilseed rape. Weeds and pests were evenly controlled across the entire trial area with herbicides and insecticides recommended by Institute of Plant Protection, National Research Institute (IOR-PIB) (herbicides: Butisan 400 SC, Command 480 EC, Leopard Extra 05 + Atpolan 80 EC;