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Full Length Research Paper

Impact of secondary metabolites and related enzymes in flax resistance and/or susceptibility to powdery mildew

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Changes in various physiological defenses including secondary metabolites, proline, total soluble protein and antioxidant enzymes were investigated in leaves and stems of 18 flax lines either resistant or susceptible to powdery mildew. The results showed that the total alkaloids content in flax stems was significantly increased in resistant lines when compared with resistant parent, but significantly decreased in susceptible lines and susceptible parent when compared with resistant parent. Stems of the resistant flax lines had a higher content of total phenols than susceptible ones upon pathogen challenge. Infection with powdery mildew significantly increased peroxidase, polyphenol oxidase and catalase activities in leaves of flax lines when compared with either resistant or susceptible parents as well as proline content. This increase was much pronounced in tolerant lines than resistant lines. Total soluble protein content in flax leaves increased significantly in resistant lines when compared with resistant parent but decreased significantly in susceptible lines when compared with resistant parent.

Key words: Flax, antioxidant enzymes, phenol, proline, secondary metabolites, powdery mildew.

INTRODUCTION

Flax (*Linum usitatissimum* L.) is considered as the major bast fiber crop in Egypt and ranks second just after cotton (seedy fiber) in relation to the cultivated area and its economic importance. Flax is cultivated in Egypt for both fiber and seed yields (dual purpose).

Powdery mildew (PM) is a foliar disease that attacks flax and is caused by *Odium lini* Skoric. The fungus attacks all aboveground parts of flax cultivars. In Egypt, it occurs wherever flax is cultivated when moisture condition and temperature are favorable. Early infections may cause severe defoliation of flax plant and reduce yield and quality. The symptoms are characterized by a white powdery mass of mycelia that starts as small spots and rapidly spreads to cover the entire leaf surface. Currently this disease is considered as the most common, conspicuous, widespread and easily recognized foliar disease of flax in Egypt.

Plants respond to pathogen attack or elicitor treatments by activating a wide variety of protective mechanisms designed to prevent pathogen replication and spread. The defense mechanisms include production of reactive oxygen species (ROS) (De Gara et al., 2003); alterations in the cell wall constitution; accumulation of secondary metabolites (Agrios, 2005); activation and/or synthesis of defense peptides and proteins (Castro and Fontes, 2005).

The involvement of phenols as secondary plant metabolites in plant disease resistance is based to a large extent on their cytotoxicity. This is associated with

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Abbreviations: ROS, Reactive oxygen species**; POX,** peroxidase**; SOD,** superoxide dismutase**; CAT**, catalase; **APX,** ascorbate peroxidase**; PPO**, polyphenol oxidases.

their oxidation products. Phenolics present in healthy, uninfected plant tissues, as antimicrobial compounds, that inhibit the growth of fungi may include simple phenols, phenolic acids, flavonols, some isoflavones and alkaloids (Mohammadi and Kazemi, 2002). Phenolics seem to inhibit disease development through different mechanisms involving the inhibition of extracellular fungal enzymes (cellulases, pectinases, laccase and xylanase), inhibition of fungal oxidative phosphorylation, nutrient deprivation (metal complexation and protein insolubilisation), the inhibition of both spore germination and mycelial growth of different pathogenic fungi and antioxidant activity in plant tissues (Chérif et al., 2007).

The production of reactive oxygen species (ROS) is one of the earliest cellular responses following successful pathogen recognition. The generation of ROS such as the superoxide anion ($O^{2.-}$) and hydrogen peroxide (H_2O_2) are a common event associated with normal plant biochemical processes (Zhao et al., 2005) and also causes oxidative damage through actions such as lipid peroxidation with membrane destruction, protein inactivation and DNA mutation (Torres et al., 2006).

Various antioxidant enzymes such as peroxidase (POX), superoxide dismutase (SOD), catalase (CAT), polyphenol oxidases (PPO) and ascorbate peroxidase (APX) participate in ROS metabolism during the pathogen attack. POXs may be some of the elements of the defense systems that are stimulated in plants in response to pathogen infection like *Fusarium oxysporum* (Morkunas and Gemerek, 2007).

Peroxidases (POXs, EC 1.11.1.7; donor: H_2O_2 oxidoreductase) are oxido-reductive enzymes that participate in the wall-building processes such as oxidation of phenols, suberization and lignifications of host plant cells during the defense reaction against pathogenic agents. One of the important physiological roles of POXs is the synthesis of cell-wall polymers (lignin and suberin), which constitute physical barriers for both biotic and abiotic stresses (Quiroga et al., 2000), which might confer the plant with high rigidity. Enhanced POX activity has been correlated with resistance in rice (Young et al., 1995) and sugarcane (McGhie et al., 1997) following the inoculation with phytopathogens.

Polyphenol oxidases (PPOs, EC1.14.18.1; monophenol, 3,4-L-dihydroxyphenylalanine: oxygen oxidoreductase) play an important role in plant defense via the oxidation of endogenous phenolic compounds into o-quinones, which are toxic to invading pathogens and pests, and PPO activity in plants increases upon fungal infections (Mohammadi and Kazemi, 2002).

Accumulation of proline occurs in response to many abiotic stresses including drought, salinity and frost as well as biotic stresses such as pathogen infection (Slama et al., 2006).

The aim of this study was to determine the changes in secondary metabolites of flax leaves and stems in response to powdery mildew infection.

MATERIALS AND METHODS

Flax cvs., that is, Wildon (P2) as tolerant parent and Cortland (P3) as the sensitive one were crossed; F_1 was self pollinated to generate F_2 seeds. F_2 individuals were subjected to PM infection; disease incidence and severity were rated as follows: disease incidence, plants showing a single spot of infection; and disease severity, percentage of infected leaves on the plant. Individual plants showing disease severity of 0 to 10% were selected as resistant, while plants showing 90 to 100% disease severity were selected as susceptible. Flowers of selected plants were bagged and left for self pollination, seeds of each individual selected plant were harvested separately and planted at the next generation. The same selection criteria were used for five successive seasons; as a result, several lines with constant response to infection were generated (either resistant or sensitive). F_7 lines were used for this study. 12 resistant lines and five susceptible ones along with their respective tolerant and susceptible parent were used. The selected lines were grown in a pot trial in three replicates and left out doors during the winter seasons of 2008 to 2009. Natural infection occurred, samples were obtained at vegetative stage (65 days after germination) and the following parameters were tested. Flax lines were considered resistant when showing disease severity of 0 to 10%, while lines showing 90 to 100% disease severity were selected as highly susceptible.

Determination of soluble protein

2 g of fresh sample were homogenized in cold phosphate buffer (0.05 M at pH 6.5). The homogenate was centrifuged at 1000 rpm for 10 min. The filtrate was completed to a known volume. The total protein content of plant tissue was estimated according to the method of Bradford (1976) and was measured at 595 nm using a spectrophotometer.

Determination of antioxidant enzymes

Peroxidase (EC 1.11.1.7) and polyphenol oxidase (EC 1.14.18.1) was assayed following the method described by Kar and Mishra (1976). The colour intensity was read at 430 nm, and the enzyme activity was expressed as the change in the optical density/gram fresh weight/hour.

Catalase (EC 1.11.1.6) was assayed following the method of Kar and Mishra (1976). The colour intensity was read at 240 nm. Catalase activity was expressed as µmole H_2O_2 destroyed/gram fresh weight/hour.

Determination of total proline

The total proline of fresh leaves was estimated according to Bates et al. (1973). The absorbance was determined at 520 nm. The concentration of proline was determined as µg/g fresh leaves.

Determination of total phenols

Levels of soluble phenols in the stems of flax lines were determined in accordance with Dihazi et al. (2003). The absorbance of the developed blue colour was read at 725 nm. Tannic acid was used

Table 1. Effect of powdery mildew on secondary metabolites, total soluble protein and proline contents in different lines of flax stems.

Line	Disease reaction	Total phenol (mg tannic acid/g)	Alkaloids (mg/g)	Total soluble protein (mg/ml)	Proline (µg / g fresh weight)
1	R Parent	3.20^{d-h}	14.67^{efg}	39.11^{d-g}	1.94^{fgh}
2	R	3.50^{d-g}	29.67^{a}	42.33^{bcd}	2.70^{e}
3	R	3.36^{d-h}	21.00^{bcd}	45.11^{b}	5.67^{b}
4	R	5.41^{ab}	21.67^{bcd}	44.33^{b}	6.46^{a}
5	R	4.12^{cde}	18.00^{cde}	50.08^{a}	3.54^{d}
6	R	4.93^{abc}	17.33^{def}	41.11^{b-e}	5.32^{bc}
7	R	4.32^{bcd}	20.33^{bcd}	44.04^{bc}	2.21^{efg}
8	R	3.53^{d-g}	22.33^{b}	44.14^{b}	2.19^{fg}
9	R	5.53^{f-i}	22.33^{b}	42.11^{bcd}	2.44^{ef}
10	R	6.03^{a}	18.33^{cde}	39.88^{c-f}	2.20^{efg}
11	R	4.34^{bcd}	17.00^{fgh}	44.62^{B}	3.54^{d}
12	R	3.66^{def}	19.67^{bcd}	42.27^{bcd}	4.85^{c}
13	H.S Parent	2.88^{e-i}	13.67^{fgh}	21.10^{i}	1.47^{h}
14	S	1.81^{ij}	11.00^{ghi}	35.40^{gh}	1.72^{gh}
15	S	1.75^{hij}	13.67^{fgh}	35.82^{fgh}	1.46^{h}
16	S	2.37^{g-j}	9.67^{i}	36.78^{fg}	2.28^{ef}
17	S	1.41^{j}	10.00^{hi}	37.72^{efg}	2.26^{ef}
18	S	2.59^{f-j}	10.00^{hi}	32.01^{h}	2.15^{fg}
L. S.D at 5%		1.26	3.76	4.2030	0.5087

Different letters indicate a significant difference according to Duncan's multiple range test. R, resistant; S, susceptible.

as standard and the amount of soluble phenols was expressed as mg tannic acid/g dry weight.

Determination of alkaloids

Alkaloids were measured according to the method described by Harbone (1973). The weight of alkaloids was then expressed as mg/g dry weight.

Statistical analysis

All data were subjected to statistical analysis, and means were compared by Duncan's multiple range test using Mstat C commuter package.

RESULTS

Changes in secondary metabolites

Table 1 shows that the total alkaloids and phenols content in flax stems increased significantly in resistant lines when compared with resistant parent but decreased significantly in susceptible lines and susceptible parent when compared with resistant parent.

Changes in proline and protein content

Table 1 shows that proline markedly increased in resistant and susceptible lines as compared to resistant and susceptible parents. This increase was much pronounced in tolerant lines than resistant lines. Table 1 also shows that total soluble protein content in flax leaves increased significantly in the resistant lines as compared to resistant parent but decreased significantly in susceptible lines when compared with resistant parent.

Changes in antioxidant enzymes

Organisms protect themselves against oxidative stress by the synthesis of various antioxidant enzymes. The results in Table 2 showed that infection with powdery mildew significantly increased peroxidase, polyphenol oxidase and catalase activities in the leaves of flax lines as compared with resistant and susceptible parents.

DISCUSSION

Changes in secondary metabolites

Plants have the ability to synthesize a large number of aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most are secondary metabolites, which may be in the form of simple phenols and phenolic acids, flavonoids and alkaloids. These

Table 2. Effect of powdery mildew on antioxidant enzymes in leaves of different flax lines.

Line	Disease reaction	Polyphenyl oxidase (activity/g fresh weight/h)	Peroxidase (activity/g fresh weight/h)	Catalase (activity µM H_2O_2/ g fresh weight/h)
1	R Parent	15.26^{d}	9.14^{fg}	26.20^{i}
2	R	18.11^{g}	10.16^{fg}	27.56^{g}
3	R	19.01^{hij}	22.08^{bcd}	28.55^{def}
4	R	15.78^{ij}	25.24^{a}	28.68^{cdef}
5	R	18.03^{f}	21.44^{cd}	27.48^{g}
6	R	18.70^{g}	20.16^{d}	28.88^{cde}
7	R	19.04^{g}	11.63^{fg}	30.96^{a}
8	R	17.05^{e}	23.00^{abc}	29.56^{b}
9	R	19.76^{k}	24.56^{ab}	27.28^{g}
10	R	16.63^{hi}	20.84^{cd}	27.36^{g}
11	R	18.54^{j}	9.82^{fg}	27.39^{g}
12	R	21.04^{b}	15.66^{e}	29.03^{bcd}
13	H.S Parent	12.47^{c}	4.61^{i}	25.48^{j}
14	S	12.91^{j}	4.62^{i}	26.71^{hi}
15	S	12.03^{a}	5.86^{hi}	27.08^{gh}
16	S	13.94^{c}	6.05^{hi}	28.19^{f}
17	S	14.70^{g}	8.03^{gh}	28.44^{ef}
18	S	13.34^{hi}	6.51^{hi}	29.23^{bc}
L. S.D at 5%		0.091	2.16	0.550

Different letters indicate a significant difference according to Duncan's multiple range test. R, resistant; S, susceptible.

substances serve as plant defense mechanisms against predation by insects, herbivores and microorganisms. Different studies showed that there are often large increases in phenolic synthesis in plants after attack by plant pathogens (De Ascensao and Dubrey, 2003).

In resistant plants, phenolic based defense responses are characterized by the early and rapid accumulation of phenolics at the infection site, resulting in the effective isolation of the pathogen. Results from many studies suggest that esterification of phenols to cell wall materials and the accumulation and deposition of phenols in and on cell walls is usually considered as an increase in resistance to fungal hydrolytic enzymes as well as a physical barrier against fungal penetration (Stadnik and Buchenauer, 2000).

Changes in proline and protein content

Proline markedly increased in resistant and susceptible lines as compared with resistant and susceptible parents. This increase was much pronounced in tolerant lines than resistant lines. These results are in agreement with Ewa et al. (2009) who found that proline concentration peaked in frost- and snow mould resistant genotype 561 and were 25 times higher in comparison with the control plants and 5 times higher than that of the susceptible genotype 621. Also, Grote et al. (2006) found proline index, the indicator of predisposition in tomato plants to *Phytophthora nicotianae* infection as influenced by abiotic stresses.

Plant pathogens such as bacteria and fungi elicit the synthesis of host proteins which help in restricting the multiplication and spread of pathogens in the healthy tissue (Datta et al., 1999). Also, Agnieszka and Iwona (2003) showed that the presence of pathogenesis-related (PR) proteins, such as chitinase, β-1,3-glucanase or thaumatin in the tissue infected by pathogens, positively correlates with the plant resistance to micro-organisms.

Changes in antioxidant enzymes

POX activity was noted to increase significantly in resistant lines as compared to resistant parent and in susceptible lines as compared to susceptible parent. These results are in agreement with Chérif et al. (2007) who found a significant increase in POX specific activity in both resistant (Wang-shuibai) and susceptible (Falat) wheat cultivars following the inoculation with *Fusarium graminearum* conidia. PPO markedly increased in resistant and susceptible lines as compared with resistant and susceptible parents, while in susceptible lines, PPO significantly decreased as compared to the resistant parent. These results are in agreement with Mohammadi and Kazemi (2002) who found that PPO specific activity significantly increased in wheat heads of resistant and susceptible cultivars following the inoculation with *F.*

graminearum conidia. In addition, Arfaoui et al. (2005) found that pretreatment of chickpea with rhizobium increased significantly the levels of peroxidase and polyphenol oxidase and total phenolics. These increases were the most effective, reducing *Fusarium* wilt development. POX and PPO are important in the defense mechanism against pathogens, through their role in the oxidation of phenolic compounds to quinines, causing increase in antimicrobial activity. Therefore, it may be directly involved in stopping pathogen development, accelerating the cellular death of cells close to the infection site, preventing the advance of infection and/or by generating a toxic environment which will inhibit the growth of the pathogen inside the cells.

CAT activity was increased markedly in flax leaves of resistant and susceptible lines. This increase was much pronounced in tolerant lines than in the resistant lines. This increase in catalase activity may provide its protection from oxidative damage by rapid removal of H_2O_2. These results are in agreement with El- Khallal (2007) who found that the activity of antioxidant enzymes in leaves under *Fusarium oxysporum* infection increased and might be effective in scavenging mechanism to remove H_2O_2 and $O_2^{\cdot-}$ produced in leaves.

The obtained results in this study led to the conclusion that the total alkaloids, phenols and total soluble protein content in flax stems significantly increased in resistant lines but decreased in susceptible ones and susceptible parent when they were both compared with the resistant parent. The activity of peroxidase, polyphenyl oxidase, catalase enzymes as well as proline content were significantly increased in powdery mildew infected leaves of flax lines as compared with either resistant or susceptible parents.

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