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Chitinase isozymes induced by TYMV and *Leptosphaeria maculans* during compatible and incompatible interaction with *Brassica napus*

B. PROFOTOVÁ*, L. BURKETOVÁ**[1], and O. VALENTOVÁ*

*Institute of Chemical Technology Prague, Technická 5, 16628 Prague, Czech Republic**
*Institute of Experimental Botany CAS, Na Karlovce 1a, 16000 Prague, Czech Republic***

Abstract

Accumulation of extracellular chitinases in *Brassica napus* plants infected with *Turnip yellow mosaic virus* (TYMV) and fungal pathogen *Leptosphaeria maculans* was studied in both compatible and incompatible interaction. Analysis of apoplast fluid by means of non-denaturing anodic and cathodic PAGE followed by in-gel detection of chitinase activity revealed a number of chitinase isozymes. TYMV induced 8 acidic and 4 basic isozymes in a systemic way. Except for one acidic and one basic isozyme, all other chitinases were also constitutively present in low amounts in mock inoculated control. In TYMV systemically infected plants, chitinases were detected in leaves expressing symptoms as well as in symptomless ones. Both virulent and avirulent *L. maculans* isolates induced production of chitinase isozymes in cotyledons in a time dependent manner. Some of them were present in plants constitutively and their content increased after inoculation. Three of five acidic and two of three basic isozymes responded to *L. maculans* infection. Chitinases started to accumulate before symptom appearance. First two acidic isozymes were detected 24 h after inoculation. The difference between compatible and incompatibe interaction reflected two basic isozymes.

Additional key words: oilseed rape, plant defence, PR-proteins, *Turnip yellow mosaic virus.*

Introduction

Plants have evolved a number of defence mechanisms to protect themselves against pathogens. Among them the role of pathogenesis-related proteins (PR-proteins), which are accumulated mostly in intercellular space upon infection, has been on focus of plant pathologists for many years. Induction of plant proteins by pathogen infection was firstly described in tobacco by Van Loon and Van Kammen (1970). Since then similar proteins were reported in a number of plant species, and their important biological roles interfering with pathogen development were described. Currently PR-proteins are subdivided into 17 families according to their properties and functions. Number of them are enzymes such as chitinases, β-1,3-glucanases, peroxidases, ribonucleases, proteases but also protease inhibitors, lipid transfer proteins, defensins and thionins were identified (Zhang *et al*. 1995, Wei *et al*. 1998, Datta *et al*. 1999, Van Loon and Van Strien 1999, Okushima *et al*. 2000, Baysal *et al*. 2005). Some of them act even against herbivore attack.

It was assumed that the expression of *PR* genes is dependent on the establishment of incompatible interaction, *i.e*. on the interaction of avirulence gene (*Avr*) products of the pathogen with resistance gene (*R*) products of the host plant. This combination results in a hypersensitive reaction, which localizes the pathogen at the site of infection (Antoniw *et al*. 1985). Despite these early findings, PR-proteins have been found also in leaves displaying non-necrotic symptoms, which indicate that their presence corresponds rather with plant resistance than symptom expression (Cole *et al*. 2001).

In order to gain information on differences in a defence response of *Brassica napus* in both compatible and incompatible interaction with pathogen, the viral and fungal pathogen were used in our study. *Turnip yellow mosaic virus* (TYMV) represents a world-wide pathogen of winter oilseed rape and a number of *Brassica* spp. (Špak and Kubelková 2002). The virus spreads systemically from the site of infection causing vein clearing and mosaic or blotching on upper leaves

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Abbreviations: PAGE - polyacrylamide gel electrophoresis, R_f - relative mobility of proteins, TYMV - *Turnip yellow mosaic virus*.
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[1] Corresponding author; fax (+420) 224310113, e-mail: burketova@ueb.cas.cz

(Matthews 1980). In contrast, ascomycete *Leptosphaeria maculans* (anamorph *Phoma lingam*), facultative necrotroph, initially colonizes mesophyll during the first biotrophic phase, and hyphae penetrate vascular strands. Subsequent stem colonization constitutes the necrotrophic phase that results in the formation of collar canker (Hammond *et al*. 1985, Howlett *et al*. 2001, Howlett 2004).

In this paper, we focused on accumulation of chitinase isozymes, which are supposed to be involved in a defence response especially against fungal pathogens.

Materials and methods

Plants: Oilseed rape (*Brassica napus* L.) cvs. Lirajet and Columbus seeds were sown directly into *Perlite* and cultivated hydroponically in a half strength Steiner's solution (Steiner 1984) in a cultivation room with day/night temperature 24/18 °C and 16-h photoperiod (photon flux density 60 μmol m^{-2} s^{-1}).

Pathogens and inoculation: TYMV was propagated on *Sinapis alba* L. Inoculum was prepared by homogenization of leaves showing typical symptoms in 100 mM phosphate buffer pH 7.5 (1 g per 20 cm^3). 3-week-old oilseed rape plants of cv. Lirajet were mechanically inoculated using a glass spatula on one fully developed lower leaf, which was dusted by carborundum beforehand. Control plants were mock inoculated with the buffer used for inoculum preparation.

Isolates JN2 (v23.2.1) and JN3 (v23.1.3) of *Leptosphaeria maculans* (Balesdent *et al*. 2001) were grown on Campbells 10 % V8 juice agar at 25 °C in dark for 14 d. Plates were subsequently transferred under fluorescent light for 10 d (24/18 °C; 16-h photoperiod) to induce pycnidia formation. Pycnidiospore suspensions were prepared by flooding the plates with sterile distilled water and scrapping the surface with a glass rod to release spores. After filtration through a cotton-wool, the spores were counted in haemocytometer and suspension concentration adjusted to 1×10^7 spores cm^{-3}.

Cotyledons of 10-day-old plants of oilseed rape cv. Columbus were punched with a sterile needle (two holes per cotyledon) and inoculated by applying 10 mm^3 droplets of pycnidiospore suspension of *L. maculans* at wounded sites (Koch *et al*. 1991). Control plants were mock inoculated in the same way using sterile distilled water instead of spore suspension. Plants were kept at higher humidity (covered by plastic sheet) for 24 h. Emerging first true leaves were continuously removed to prevent cotyledons from senescence.

Intercellular washing fluid isolation: Intercellular washing fluid (IWF) was collected at defined sampling times by modified method according to Pierpoint (1987). Cotyledons of 60 plants or leaves of 30 plants, respectively, were cut off, dipped into an extraction buffer (25 mM Tris-HCl, pH 7.8) and infiltrated under vacuum for a few minutes. Subsequently, the leaves were tissue dried, gently rolled, put into the barrel of a 25 cm^3 syringe and placed in a centrifuge tube. IWF was recovered from apoplast by centrifugation at low speed (1000 *g*, 4 °C, 10 min) and stored at -20 °C until analysis.

Gel electrophoresis and "in-gel" chitinase activity detection: The extracellular proteins were separated using discontinuous non-denaturing polyacrylamide gel electrophoresis (PAGE) according to Davis (1964) in 10 % resolving gels using Tris-glycine buffer (pH 8.8). For analysis of acidic and basic proteins both anodic and cathodic mode of electrophoreses were used. The gels were run using the *Mighty Small II* apparatus (*Hoefer Scientific Instruments*, San Francisco, USA) at constant current 15 mA and 25 mA per gel for acidic and basic proteins, respectively. After electrophoresis chitinase activities were detected. The amount of proteins loaded to each well corresponded to IWF obtained from approximately 0.4 g of fresh leaf tissue.

Chitinase activity was detected after PAGE in overlaying substrate gel containing 1 % glycolchitin as described by Trudel and Asselin (1989). The gels were incubated for 2 h at 37 °C and stained with 0.01 % calcofluor. Chitinase activity appeared after washing out the digested substrate under UV light as dark bands on the calcofluor stained gel. Intensity of chitinase bands on zymogram was evaluated using *ImageMaster*™, *Total*Lab, version *2.0* (*Amersham Pharmacia Biotech*, Uppsala, Sweden).

Chemicals were purchased from *Sigma-Aldrich* (St. Louis, USA).

Results

TYMV infection of *B. napus* and chitinase accumulation: When 3-week-old plants were TYMV inoculated on one leaf, typical symptoms as mottling and mosaic has developed within next two weeks on upper leaves (Fig. 1), however, some of the leaves remained symptomless. Intercellular washing fluid isolated from locally and systemically infected leaves, both with and without symptoms, was subjected to non-denaturing PAGE with subsequent "in-gel" detection of chitinase activity (Figs. 2, 3). In all groups of leaves a number of bands with chitinase activity appeared. Except for one acidic (R_f 0.44) and one basic (R_f 0.24) isozymes, all others were constitutively present in low amounts also in leaves of control plants and their content strongly

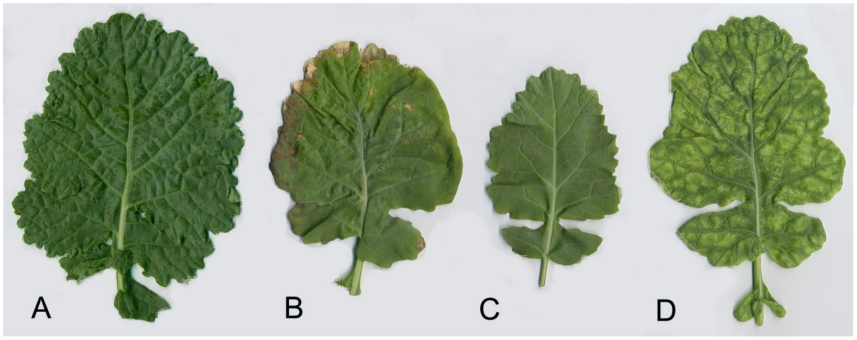

Fig. 1. TYMV infection on *B. napus* cv. Lirajet. TYMV was inoculated on one lower leaf of 3-week-old plants. Within two weeks mosaic symptoms developed on upper young leaves, some leaves of plants remained without symptoms. Mock inoculated control leaf (*A*), locally infected (inoculated) leaf (*B*), systemically infected symptomless leaf (*C*), systemically infected leaf displaying symptoms (*D*).

increased after TYMV infection (acidic: R_f 0.13, 0.19, 0.24, 0.28, 0.38, 0.47, and 0.53; basic: R_f 0.10, 0.22, 0.54). All of the chitinase isozymes were detected regardless the presence or absence of symptoms. Even though no symptoms were visible, one acidic isozyme (R_f 0.13) was found in addition to those detected in symptom displaying leaves.

***L. maculans* infection of *B. napus* and chitinase accumulation:** No necroses were recorded on cotyledons until one week post inoculation with both isolates. The symptoms started to be pronounced after at least 10 d (Fig. 4). Avirulent isolate JN3 induced hypersensitive response at the site of infection, which resulted in reduced necroses development. On the contrary, virulent isolate of the fungus JN2 grew quickly from the site of inoculation, and spread all over the whole cotyledon within three weeks.

The conspicuous variation in the pattern of extracellular chitinase isozymes was observed in a time course experiment (Figs. 5, 6*A*,*B*). Although the first symptoms of *L. maculans* infection became evident at day 10 after inoculation at earliest, two acidic chitinase isozymes (R_f 0.38 and 0.44) appeared already after 24 h, and their content increased during the time of experiment. 14-d-old infected cotyledons accumulated 5 acidic and 3 basic isozymes, either newly formed or with strongly increased amounts, comparing to mock inoculated control. Detection of chitinase activity in the gel revealed that

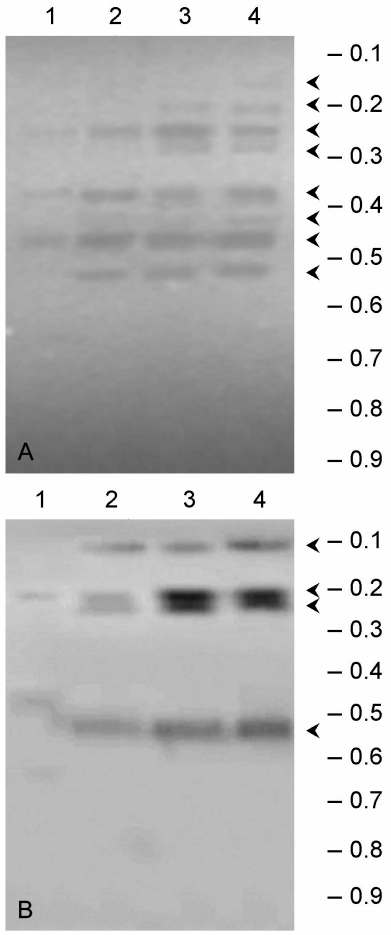

Fig. 2. Accumulation of extracellular chitinases in leaves of *B. napus* cv. Lirajet after TYMV infection. Two weeks after inoculation, mock treated leaves (1), inoculated leaves (2), symptom displaying leaves (3) as well as leaves without symptoms (4) were separately collected, IWF isolated, and extracellular proteins analyzed. Acidic and basic proteins were separated by native PAGE on 10 % resolving gel. Acidic electrophoresis was performed at 15 mA per gel, basic PAGE was run at 25 mA for 3 h. "In gel" activities of acidic (*A*) and basic (*B*) chitinases were detected after PAGE in overlaying substrate gel containing glycol chitin (1 %). Comparing to mock-inoculated plants (1), TYMV induced both acidic (*A*) and basic (*B*) chitinase isozymes in inoculated, mosaic, and symptomless leaves (pointed by *arrows*). The most pronounced difference in chitinase activity pattern was found in mosaic leaves (3) as well as in leaves without symptoms (4). The scale indicates relative electrophoretic mobility (R_f) of chitinase isozymes. Changes reported here were observed consistently in at least 3 successive experiments.

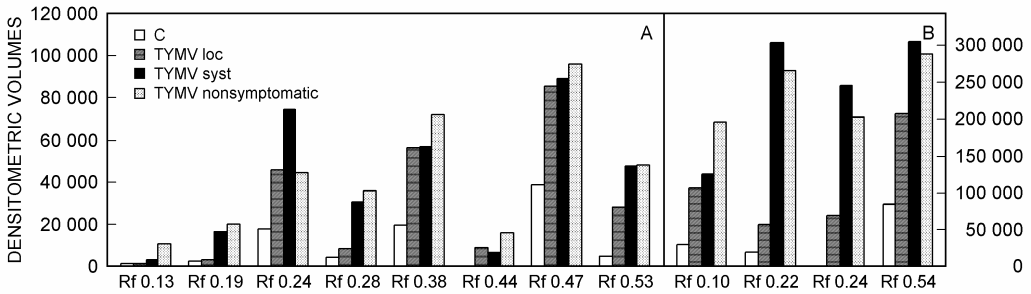

Fig. 3. Intensity of chitinase bands reflecting TYMV infection were quantified using *ImageMaster*™. C - mock inoculated control, TYMV loc - TYMV inoculated leaves, TYMV syst - systemically infected leaves with expressed symptoms, TYMV nonsymptomatic - leaves without symptoms originated from TYMV systemically infected plants; *A* - acidic chitinases, *B* - basic chitinases.

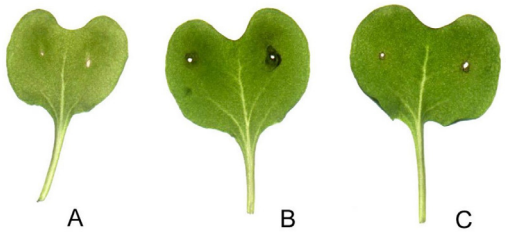

Fig. 4. Cotyledons of *B. napus* cv. Columbus infected with avirulent (JN3 - *on the left*) and virulent (JN2 - *on the right*) isolates of *L. maculans* 14 dpi. Plants were inoculated with spore suspension at wounded site caused by perforation of cotyledons, control plants were mock inoculated (*A*). The symptoms usually appeared visible after 10 d. Incompatible interaction (*C*) was characterized by small necrotic lesions development that formed as a hypersensitive response of the plant. Compatible interaction (*B*) resulted in a severe plant tissue necrotization leading to the destruction of cotyledon within three weeks.

except for one acidic (R_f 0.40) and two basic (R_f 0.42, 0.29) chitinase isozymes, most of the others were present in small amounts also in non-infected cotyledons, where often appeared at later stages of the experiment, which could be explained by cotyledons senescence. Acidic isozyme of R_f 0.57 was detected only in 11-d-old (*i.e.* 24 h after inoculation) plants and not later on indicating its inducibility by mechanical injury during inoculation. Both acidic (R_f 0.38, 0.40, 0.44) and basic (R_f 0.29, 0.42) isozymes were prominent as a response to *L. maculans* infection. The difference between compatible and incompatible interaction was noticeable at a very early stage (24 h) of infection regarding acidic isozyme (R_f 0.38). The basic isozymes (R_f 0.29, 0.42) reflected defence response to avirulent isolate (JN3) one week post inoculation, no difference between the two isolates was found afterwards.

Discussion

Chitinases belong to the group of hydrolytic enzymes suspected to retard growth of fungi, in whose chitin is the main constituent of cell wall. Beside the antifungal role, their connection with various kinds of stresses and developmental cues has bee reported (Collinge *et al.* 1993, Datta *et al.* 1999). Some of chitinases can be produced by plant either as a specific response to particular pathogen or non-specifically as a general defence reaction, and even by non-pathogenic plant growth-promoting rhizobacteria (Thangavelu *et al.* 2003). To investigate their inducibility in *B. napus*, two diverse pathogens were used in our experiments. While TYMV represents an obligate parasite fully dependent on the host metabolism, *L. maculans* as a facultative necrotroph, after the first biotrophic phase, utilizes plant constituents *via* its own enzymatic machinery (Howlett *et al.* 2001).

Analysis of extracellular proteins provides a useful tool for the study of plant defence response as was shown by Robertson *et al.* (1997), Burketová *et al.* (1999), Šindelářová and Šindelář (2001) and Gau *et al.* (2004). This proteomic approach enables to cover a wider range of induced proteins than focusing on expression of particular proteins or genes. The isolation of IWF from *B. napus* plants is awkward comparing to some other plant species because of thick wax layer and rigid tissues. Despite this inconvenience we have decided to use this method on the base of our previous experience. PAGE of IWF provides much clearer pattern of proteins, which are not drown in a plenty of cytosolic ones present in homogenate. Detailed localization of these induced proteins was not the subject of this work and should be studied in future.

Even though chitinases have been detected in virus infected plants of various species, there are no data available on PR-proteins induction by virus infection in *B. napus*. Principally, the production of PR-proteins is a downstream event from recognition of the virus by host plant based on interaction of *Avr* and *R* genes products. This kind of response is characterized by hypersensitive reaction and onset of systemic acquired resistance (SAR) at distant parts of the plant. As was reported earlier, some of PR-proteins produced in plants are induced in

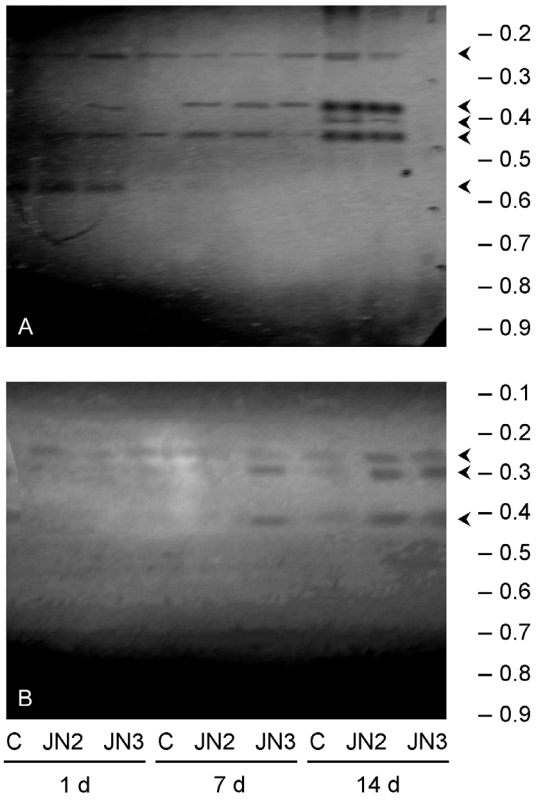

Fig. 5. Accumulation of extracellular chitinases in cotyledons of *B. napus* cv. Columbus after inoculation with avirulent (JN3) and virulent (JN2) isolates of *L. maculans*. Cotyledons were collected 1, 7, and 14 d after inoculation. IWF was isolated, and extracellular proteins analyzed. Acidic and basic proteins were separated by native PAGE on 10 % resolving gel. Acidic electrophoresis was performed at 15 mA per gel, basic PAGE was run at 25 mA for 3 h. "In gel" activities of acidic (*A*) and basic (*B*) chitinases were detected after PAGE in overlaying substrate gel containing glycol chitin (1 %). Analysis of extracellular proteins in cotyledons revealed *de novo* or increased synthesis of both acidic and basic chitinase isozymes, which become evident 7 d after inoculation. At least three acidic isozymes were present constitutively. The scale indicates relative mobility (R_f) of chitinase isozymes. Changes reported here were observed consistently in at least 3 successive experiments.

dependence on symptoms development, *e.g.*, PR-4d in *Phaseolus vulgaris* was induced only in cultivars developing necrotic but not mosaic symptoms (Sehgal *et al*. 1991). In our experiment, *B. napus* cv. Lirajet responded to TYMV infection by non-necrotic symptoms as mottle and mosaic, some of the leaves remained symptomless. Chitinase isozymes were found in all leaves including those without symptoms, which indicate their systemic induction by the virus. Owing to the improbable role of these enzymes in defence against virus; we can assume that their presence in leaves is rather a part of unspecific defence response.

Comparing to *B. napus* response to TYMV infection, chitinase production in cotyledons of cv. Columbus inoculated with *L. maculans* was less pronounced. In the pathosystem studied, both compatible and incompatible interactions were designed using *L. maculans* isolates, which either have *Avr* genes (isolate JN3 comprises AvrLm5-6-7-8 and AvrLm 1) interacting with *R* genes in cv. Columbus (Rlm1) or not (isolate JN2 comprises AvrLm5-6-7-8) (Balesdent *et al.* 2001). The lower number of induced chitinase isozymes in cotyledons can be explained by difference in defence capacity of cotyledons and true leaves, which were used in TYMV experiments or by diverse pathogens infecting the plant. Detection of inducible basic chitinase isozymes in our experiment is in accordance with results of other authors. Rasmussen *et al*. (1992), detected *L. maculans* induced basic chitinase serologically related with sugar beet chitinase isozyme IV, which was expressed differently in susceptible and resistant cultivars, and was localized in the site of infection. Similarly, isolates of *L. maculans* with different virulence were studied in both resistant and susceptible cultivars by Dixelius (1994). Immunoblot of proteins induced after inoculation revealed transient expression of PR-proteins cross-reacting with antibodies raised against PR-2, PR-Q and PR-S, which were associated with hypersensitive response of tobacco against *Tobacco mosaic virus*. Interaction of *B. napus* and *L. maculans* was also studied by means of molecular methods. Fristensky *et al.* (1999) sequenced 277 cDNA clones from the library derived using mRNA from *B. napus* leaves inoculated with an avirulent isolate of

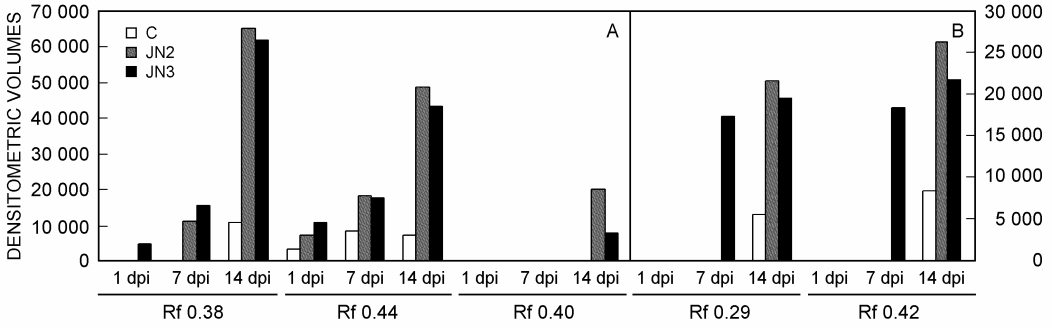

Fig. 6. Intensity of three acidic (*A*) and two basic (*B*) major chitinase bands reflecting *L. maculans* infection. Chitinase bands in zymograms were quantified using ImageMaster™. C - mock inoculated control, JN2 - virulent isolate of *L. maculans*, JN3 - avirulent isolate of *L. maculans.*

L. maculans. Resultant ESTs included acidic chitinase class II (Chia1) and PR3 endochitinase class IV (Chia4). In addition, induction of chitinase class IV and PR-1a protein was demonstrated also in connection with senescence of *B. napus* leaves (Hanfrey *et al*. 1996).

To conclude, *B. napus* responds to pathogen attack by producing chitinase isozymes regardless of symptom expression. In case of TYMV infection, these enzymes are induced in a systemic manner, which is contradictory to findings of Rasmussen (1992) in *L. maculans* infected plants. When compared results of authors cited above and our results presented here, it can be suggested that *B. napus* responds to pathogen attack by a wider scale of chitinase isozymes than have been described up to now. Additional studies are required for their identification.

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Raman, S.: **Agricultural Sustainability. Principles, Processes, and Prospects.** - Food Products Press, An Imprint of The Haworth Press, New York - London - Oxford 2006. 474 pp. USD 69.95. ISBN 13: 978-1-56022-310-8.

This book contains a comprehensive survey of nearly all aspects of agricultural sustainability. The subtitle correctly indicates that the text deals with sustainability principles, processes and prospects in agriculture. Sustainable agriculture has been operated at many places on the Earth for centuries. However, it was never requested to feed some 10 billion people while ensuring "plant and animal productivity adequate to meet the needs of a growing population, ecological security and environmental quality, economic viability, social responsibility and acceptability" (p. 32). This is an absolutely new challenge not only for farmers themselves, but literately for mankind. Hence, a full-scale account on this topic as presented in the reviewed book is to be warmly welcome.

The book starts with a Foreword written by M.S. Swaminathan. I recommend all readers not to skip over these wise 3 pages. The contiguous Preface introduces the reader to the general context of sustainability and the very introduction explaining the intellectual layout, *i.e.*, organisation of the book. In fact, the text is divided into three parts as follows. Part I: Principles and paradoxes of agricultural sustainability (pp. 19 to 70) dealing also with contextual nature and conceptual framework of sustainability in agriculture. Part II: Operationalization of sustainable agriculture (pp. 71 to 264). In its 8 chapters the following topics are dealt with: resources and their conservation in nature, land management, soil quality, water management, biodiversity, energy management, and indices of agricultural sustainability. In this part, much detailed information has been included with many important values (for example global water resources and their use, ecosystem services, energy consumption, and indicators of soil quality). Although some of the statistical data are somewhat out of date referring mainly to the last decade of the 20th century, their qualified analyses and comments give sufficient evidence of their impact. Part III is entitled "Transition to agricultural sustainability" (pp. 265 to 430). Its two chapters assess challenges to global agricultural sustainability and ways how to achieve it in the near future. Last but one chapter describes "Sustainable livelihoods and rural development". The last chapter summarizes basic concepts of sustainable agriculture including its ethics. Bibliography occupies pages 431 to 458 and the book terminates with a detailed index.

As far as I am aware, this book is one of few sources of knowledge, ideas, principles, presumptions, suggestions, recommendation and hypotheses on sustainable agriculture in its broadest scope and particulars. Browsing, reading and studying its text will help understand both the term and necessity of sustainable agriculture. It is a remarkable achievement by its author. Myself, a crop physiologist, I miss just one aspect in dealing with sustainability: biology of the most important crop species as a non-replaceable aspect of future improvements aiming at reaching both sufficient food production while preserving nature ecosystems. An increase in water use efficiency, higher rates of the ratio of harvested solar energy to invested fossil energy, low input nutrient genotypes, *etc.* cannot be achieved if no better knowledge on both general and special (crop species oriented) plant biology is accomplished. But I admit, that this aspect would slightly lie outside the general scope of this book. Nevertheless, these biological aspects should be accepted as equipotent or even dominant over management or economic viewpoints.

Briefly, this book represents a valuable contribution to recent discussion on sustainability. It is written by a very competent author. Much information is offered to everybody who is professionally engaged in sustainability study, planning or management. Its diction is such that it satisfies professionals and remains understandable to non-experts. It is a pleasure for me to thank the author for such a book and congratulate him on this comprehensive and competent text.

L. NÁTR (*Praha*)