

Original Research

The Role of Pesticides in the Development of Populations of Microorganisms Colonizing the Leaves of Winter Wheat

U. Wachowska*

Department of Phytopathology and Entomology, University of Warmia and Mazury,
Prawocheńskiego 17, 10-597 Olsztyn, Poland

Received: 3 July, 2003

Accepted: 25 June, 2004

Abstract

Studies on sensitivity of microorganisms colonizing the leaves of winter wheat cultivar Almari to Amistar 250SC, Bravo 500SC fungicides and Bion 50WG plant resistance stimulator were performed in 2000-01.

The studied fungicides decreased growth of hyphae fungi colonizing the leaves of winter wheat cultivated in pots, especially 24 hours after treatment, compared to control. Created free ecological niche was inhabited by bacteria and yeast-like fungi. From leaves treatment with Bion 50WG, the number of isolated microorganisms was bigger than control.

In vitro fungicides Bravo 500SC and Amistar 250SC were very toxic to fungi *Rhodotorula* sp., *Saccharomyces* sp. and *Cryptococcus* sp. Colonizing the leaves of winter wheat. Bion 50WG plant resistance stimulator was toxic, but only in concentrations of 50 and 500 µg to *Rhodotorula* sp. Studied plant protection agents stimulated of strain *Candida albicans*.

Keywords: fungicide, microorganisms, leaf, wheat

Introduction

The leaf surface of winter wheat is a specific ecosystem inhabited by bacteria and fungi; an ecosystem which is mainly shaped by plant secretions [1, 2, 3, 4, 5, 6, 7]. Characteristic species of this ecological niche are: *Alternaria alternata* (Fr.) Keissler, *Cladosporium* spp. *Fusarium* spp., *Aureobasidium pullulans* (de Bary) Arn. [1, 2, 7]. Nevertheless, yeast-like fungi play the dominant role here; most frequently mentioned are the species that belong to the following genera: *Sporobolomyces*, *Rhodotorula*, *Candida*, *Cryptococcus* [7, 8, 9]. As far as epiphytic bacteria are concerned, the following genera are worthy of mention: *Pseudomonas*, *Bacillus*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Xanthomonas* and *Pantoea* [10, 11,

12, 13]. A homeostasis is established between the microorganisms colonizing the leaf surface; this homeostasis is put under pressure from the environment, which is both naturally occurring and anthropogenic. Plant pesticides in this system may be treated as an impermanent ecological stress. These preparations, when applied as recommended by a manufacturer, act at low or medium concentrations, which is too weak and too short-acting to produce any irreversible changes in the ecosystem [3, 14, 15]. It should be remembered, however, that fungicides are extensively used in agriculture and their microenvironmental impact is widespread [16]. Exhaustive tests for the toxicity of fungicides are being carried out on animals; nevertheless, in recent years many publications have appeared and they show alternative methods of toxicological evaluation of plant pesticides, methods that are fast and cheap [17, 18]. Yeast-like fungi, which are eukaryotic organisms, provide

*e-mail: urszula.wachowska@uwm.edu.pl

a potentially suitable model for cytotoxicological evaluation of preparations which are introduced to the agricultural environment [19, 16]. Furthermore, they are widespread, they commonly occur on leaves and they play a significant role under natural conditions, a role which has not yet been fully explained [1, 2, 3, 4, 5, 6, 7].

The present study is an attempt to determine the sensitivity of various groups of microorganisms colonizing the wheat leaf surface to plant pesticides which contain chlorotalonil, strobilurin and acibenzolar-S-methyl; it is also an attempt to use yeast-like fungi as model organisms for toxicological evaluation of the aforementioned preparations.

Materials and Methods

The Outline of the Experiment

Pot experiments were carried out in two series in 2000-01. The exact experiment was located in the Experimental Station in Tomaszkowo near Olsztyn under conditions of natural sources of foliar infections. Winter wheat, the cultivar Almari, was the object of the study. It grew in 30 cm diameter pots (20 plants/pot). The experiment was planned in a completely randomized design in four replications. At the beginning of blade spraying the plants were sprayed with plant pesticides in the recommended commercial doses. A plant resistance stimulator called Bion 50WG (acibenzolar-S-methyl) and the fungicides Bravo 500SC (chlorotalonil) and Amistar 250SC (azoxystrobin) were applied. Objects that were sprayed with sterile water provided a control.

Collecting Plant Samples

The analysis of the population of microorganisms colonizing the leaf surface of winter wheat was carried out after 24 hours (the end of growing apice (GS 29)), after 10 days (the beginning of blade spraying (GS 30)) and after 20 days the stage of the first node (GS 31) from the moment of plant pesticides application. Ten full-grown leaves were collected from each pot, always third from the top. A cumulative sample was taken from the collected leaves and next, 1 cm pieces were cut from the base part of leaf blade; then their width was measured. Different methods were adopted according to the group of the microorganisms isolated from the surface of the leaves.

Microbiological Analysis

Leaf cut-outs (15 from each combination) were rinsed in 15 ml of sterile water in 200 ml flasks. The flasks together with leaf pieces were shaken for 30 minutes in a 358S-type table shaker (frequency 170/min, amplitude 8) [5, 7]. 0.1 ml of microorganic suspension was taken from each flask, and then it was inoculated with a palp into the following substrata: nutrient agar pH 7.2 used for isolating bacteria, Martin's medium (PDA medium with rose Bengal and streptomycin) used for isolating fungi.

The experiment was planned in four replications. The microorganisms were incubated in the dark at 25°C. After the number of colonies on the plate had established, their counts were calculated per 1 cm² of the leaf surface. The results were statistically handled by means of the analysis of variance on transformed data ($y=\log(x+1)$).

The Isolation of Ballistosporic Fungi

One-centimetre leaf pieces were attached to the Petri upper plate with petroleum jelly [5]. The lower plate was filled with potato dextrose nutrient medium. The experiment was planned in four replications. The microorganisms were incubated in the dark at 25°C. After four days the grown colonies of fungi were counted four times, every 24 hours. After their number on the plate was established, their counts were calculated per 1 cm² of the leaf surface. The results were statistically handled by means of the analysis of variance on transformed data ($y=\log(x+1)$).

The qualitative analysis of the composition of the community of hyphal fungi. The qualitative composition of the population of hyphal fungi was analyzed 24 hours, 10 days and 20 days after each fungicide treatment [15]. The colonies of fungi that had different morphological features were subcultured from Martin's medium onto agar slants. The slants were incubated for 7 days in the dark at 25°C. The fungi were determined on the basis of the available keys and monographs [20, 21, 22, 23, 24, 25, 26, 27, 28].

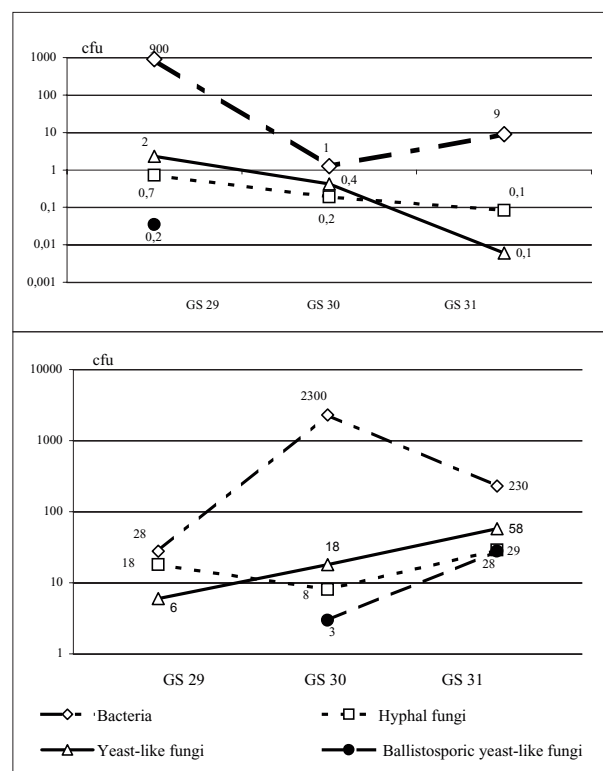


Fig. 1. Number of microorganisms colonizing the leaves of winter wheat in the years 2000 (top) and 2001 (down).

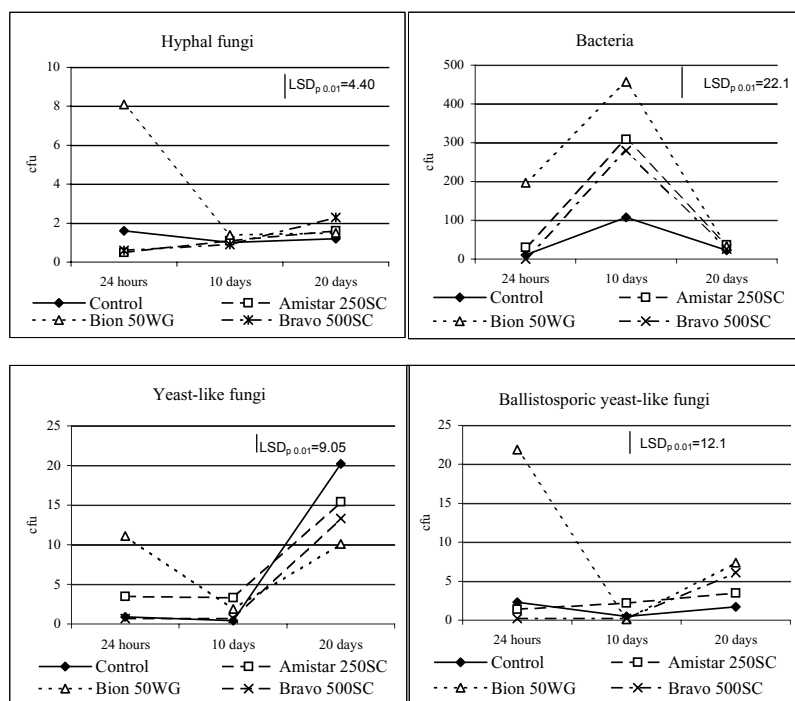


Fig. 2. The effect of pesticides on microorganisms colonizing the leaves of winter wheat (sum in years 2000-01).

The Influence of Selected Plant Pesticides on the Growth of Yeast-Like Fungi

The method of "intoxicated substrata" [29] was employed in the studies. The following concentrations of the plant resistance stimulator Bion 50WG and the fungicides Amistar 250SC and Bravo 500SC were applied: 5, 50, 500 μ l of biologically active substance in 1 ml of PDA substratum. Petri plates without the preparation provided a control. One analyzed the growth of yeast-like fungi of the genera *Cryptococcus*, *Candida* and *Rhodotorula* that were collected from leaves, and an isolate of *Candida albicans* that was collected from human oral cavity. These fungi were determined on the basis of the form of microculture and on the basis of biochemical tests used for the determination of yeast-like fungi, called API 20 C AUX [30, 31, 32]. After their number on the plate was established, their counts were calculated by means of computer image-analysis using a program called Microscan. The experiment was planned in four replications. The results were statistically analyzed and given in percentage of control combination.

Results

The composition and count of the communities of microorganisms that belong to winter wheat phyllosphere were characteristic of this ecological niche. Bacteria were dominant here (Fig. 1); in the year 2001, when the system of weather conditions was conducive to their growth, their count averaged about 2300 of colony-forming units (CFU) per 1 cm^2 of leaf surface. The community of fungi

emerged to be quite numerous and diversified. Yeast-like fungi were most frequently isolated. In the second year of studies, from 6 to 58 CFU were obtained, on average, from 1 cm^2 of a leaf of winter wheat, depending on the time of observation. Hyphal fungi and ballistosporic yeast-like fungi were few. In 2000 hyphal fungi were obtained occasionally, while ballistosporic yeast-like fungi were obtained only at the stage of growing apace (GS 29). The system of weather conditions in 2001 was more conducive to the growth of epiphytic fungi. In addition, the older the plants were, the more numerous the communities of hyphal fungi and ballistosporic yeast-like fungi became (Fig. 1).

In most cases the fungicides under study showed various direction and power of action on the studied groups of microorganisms in comparison with the preparation Bion 50WG (Fig. 2). One day after the application of fungicides on leaves the numbers of hyphal fungi and ballistosporic yeast-like fungi were definitely lower in comparison with the control, while in the case of the fungicide Bravo 500SC the same held true also for the rest of yeast-like fungi. In this observation period the resistance of plant stimulator Bion 50WG was definitely stimulating to all the studied groups of microorganisms. After 10 days from the protective treatment the count of communities of the studied epiphytic hyphal fungi was back at the control level. At that time the count of the bacteria community in combinations where the preparations were applied was definitely higher than in the control. A higher number of yeast-like fungi in comparison with the control were also observed on winter wheat leaves which were protected with the preparation Amistar 250SC. After 20 days the reductive

Table 2. The effect of preparations on the survival rate of the yeasts fungi.

| Species of fungi | Preparation | Contents of active ingredient in $\mu\text{l}/1 \text{ ml PDA}$ | | | Mean |
|---------------------------|---------------|--|----------|----------|--------|
| | | 5 | 50 | 500 | |
| | | Survival rate (% of control) | | | |
| <i>Cryptococcus</i> sp. | Amistar 250SC | 0a* | 0a | 0a | 0a |
| | Bion 50 WG | 100.0bcd | 47.7ab | 44.3ab | 64.0b |
| | Bravo 500SC | 83.0abc | 0a | 83.3abc | 55.4b |
| | Mean | 61b | 16a | 42ab | |
| <i>Candida</i> sp. | Amistar 250SC | 4.0a | 4.1a | 3.9a | 4.0a |
| | Bion 50 WG | 89.3bcd | 70.8abc | 72.0abc | 77.4b |
| | Bravo 500SC | 4.0a | 3.4a | 3.7a | 4a |
| | Mean | 32.3ab | 26.1ab | 26.5ab | |
| <i>Candida albicans</i> | Amistar 250SC | 132.3bcd | 126.3bcd | 118.7bcd | 125.8a |
| | Bion 50 WG | 165.0de | 111.7bcd | 113.7bcd | 130.1c |
| | Bravo 500SC | 151.0cde | 165.7de | 205.0e | 173.9d |
| | Mean | 149c | 123c | 146c | |
| <i>Rhodotorula</i> sp. 21 | Amistar 250SC | 1.1a | 1.8a | 0.8a | 1.2a |
| | Bion 50 WG | 109.2bcd | 120.3bcd | 109.3bcd | 112.9c |
| | Bravo 500SC | 0.7a | 0.5a | 0.5a | 0.6a |
| | Mean | 37.0ab | 40.8ab | 36.9ab | |
| <i>Rhodotorula</i> sp. 25 | Amistar 250SC | 0a | 0a | 0a | 0a |
| | Bion 50 WG | 94.7bcd | 47.9ab | 0a | 47.5b |
| | Bravo 500SC | 0a | 0a | 0a | 0a |
| | Mean | 31.6ab | 15.9a | 0a | |

* - values marked by the same letters do not differ significantly according to Newman-Kuels

the studied fungicides did not exceed 5%, irrespective of the concentration of biologically active substances in the substratum.

The plant resistance stimulator Bion 50WG had negative action on the growth of the fungus of the genus *Rhodotorula* strain 25 only at a concentration of 50 $\mu\text{g}/1 \text{ ml PDA}$, when it reduced its growth by more than 50% in comparison with the control (Table 2). The second strain which belonged to this genus grew better in the presence of plant resistance stimulator than without it. The viability of fungi of the genera *Cryptococcus* and *Candida* in the presence of Bion 50WG ranged, respectively, from 44.3 to 72.0% at its highest concentration (500 $\mu\text{g}/1 \text{ ml PDA}$) and from 47.7 to 70.8% at a lower concentration (50 $\mu\text{g}/1 \text{ ml PDA}$).

All the studied plant pesticides had a stimulating action on the growth of the *Candida albicans* colony collected from human oral cavities, at all concentrations (Table 2). This direction of action was most observable in the presence of 500 μl chlorotalonil in 1ml of PDA substratum.

The preparations Bion 50WG and Amistar 250SC had the strongest action at a concentration of 5 μl of biologically active substance in the substratum.

Discussion of Results

The leaf surface emerged as an ecological niche that was particularly sensitive to the change of atmospheric conditions. Heavy rainfall in May 2000 (almost twice as much as in perennation) caused the washing off of microbial spores [2] and organic compounds from the leaf surface of winter wheat [6]. May 2001 was warm and humid, but without heavy rainfall, which was conducive to the growth of microorganisms on leaves. Exceptionally low temperatures in the second decade of May of that year were conducive to the accumulation of psychrophilic fungi of the genus *Cladosporium* [24].

The inhibitory action of plant pesticides on hyphal fungi was considerable one day after the protective treatment. Fungi of the genus *Cladosporium* were hardly sen-

Table 3. Ecological information (date of producer).

| Ecotoxicity | Amistar 250SC | Bravo 500SC | Bion 50 WG |
|---------------------|---------------|-------------|------------|
| Toxicity to fish | 2.4 mg/l | 0.195 mg/l | 1.3 mg/l |
| Toxicity to daphnia | 0.47 mg/l | 0.882 mg/l | 2.8 mg/l |
| Toxicity to algae | 0.26 mg/l | 0.521 mg/l | 4.8 mg/l |

sitive to plant pesticides, results that are consistent with Błaszczowski's findings [1]. The applied fungicides considerably reduced the number of isolations of non-sporifying colonies, which increased the number of species of spore-forming fungi. This confirmed Błaszczowski's findings [1] that fungicides, being a stressful factor, induce sporification in fungi.

Yeast-like fungi appeared to be the commonest fungi that grow on winter wheat leaves [9]. Their function is still to be examined. It is presumed that they may, among other things, reduce the growth of typical leaf pathogens. For this reason it is important to know whether the applied fungicides limit the spectrum of non-pathogenic fungi that occur on the leaf surface [8]. High sensitivity of ballistosporic yeast-like fungi to the action of the examined fungicides seems to be not very favourable; this sensitivity had been also observed earlier [5]. The sensitivity of the rest of yeast-like fungi to the examined fungicides seems to be disputable. These fungi, indeed, appeared to be insensitive to the applied fungicides a short time after treatment, just as was the case in Mikołajska's examinations [7]. Later observations, however, show their high sensitivity to some plant pesticides.

The examinations carried out at the initial stages of wheat growth supported the thesis that bacteria are the first to appear on leaves and they are pioneers followed by yeast-like fungi and next by hyphal fungi [11]. The application of chlorotanoniol, which shows high activity on many fungi, *Ascomycetes* in particular, caused the increase of count of bacteria on leaves. Rodgers-Grey and Shaw [11] observed the same dependence.

The applied strains of yeast-like fungi (collected from leaves, in particular, of the genus *Rhodotorula*) appeared to be very sensitive to the examined fungicides under laboratory conditions. It seems possible to use these eukaryotic organisms for toxicity tests of plant pesticides [16]. Chlorotanoniol, a substance that has been used on a large scale for many years [33]), shows high toxicity to fish, daphnia, and algae (tab. 3). Its toxicity to other yeast-like fungi is also high. Azoxystrobin shows a wide spectrum of action [33]. Its toxicity to water organisms, fish, daphnia and algae is low. Definitely, the lowest ecological toxicity of Bion 50 WG was confirmed in the present study.

Strong resistance of the strain *Candida albicans* collected from a different environment to the examined preparations that are widely used in agriculture is perplexing. This

species is considered pathogenic for a human being. It can be transmitted in the environment by wild animals or farm animals [34], as well as by surface water [35]. It emerges that in the case of the analyzed plant pesticides it may be expected that they will also stimulate the growth of population of this pathogenic species in its natural reservoirs.

References

1. BŁASZKOWSKI J. The effect of foliar fungicides on the mycoflora of seeds of *Triticum aestivum* L. *Acta Mycol.* **29**, 147, **1994**.
2. BŁASZKOWSKI J. Biotic effects of microflora of lives, glumes and seeds on *Septoria nodorum* following *Triticum aestivum* treatment with fungicides. *Acta Mycologica* **30** (2), 223, **1995**.
3. BURCHARDT L. Theory and practise of ecological studies. Published by *Sorus*. (In Polish) Poznań: 140, **1994**.
4. DICKINSON C.H. Effects of ethirimol and zineb on phylloplane microflora of barley. *Trans. Br. Mycol. Soc.* **60** (3), 423, **1973**.
5. DICKINSON C. H., WALLACE B. Effects of late applications of foliar fungicides on activity of microorganism on winter wheat flag leaves. *Trans. Br. Mycol. Soc.* **16**, (1), 103, **1976**.
6. DIX N. J., WEBSTER J. Fungal ecology. Chopman and Hall. London; pp. 499, **1995**.
7. MIKOŁAJSKA J. Effect of some fungicides on winter wheat leaf microflora. (In Polish) *Zesz. Probl. Post. Nauk. Rol.* **307**, 197, **1987**.
8. BASHI E., FOKKEMA N.J. Environmental factors limiting growth of *Sporobolomyces roseus*, an antagonist of *Cochliobolus sativus*, on wheat leaves. *Trans. Br. Mycol. Soc.* **68** (1), 17, **1977**.
9. CHRUSCIAK E. The microflora of the phyllosphere. (In Polish). *Acta Mycol.* **10** (1), 173, **1974**.
10. GOODFELLOW M., AUSTIN B., DICKINSON C. H. Numerical taxonomy of some yellow-pigmented bacteria isolated from plants. *Jurnal of General Microbiology*, **97**, 219, **1976**.
11. RODGERS - GRAY B.S., SHAW M.W. The effect of incorporating straw or manure into the soil on the natural microflora of winter wheat. *Plant Pathology* **50**, 537, **2001**.
12. RUSSEL S. Microorganisms and soil life. (In Polish) pp. 302, **1974**.
13. WILSON M., LINOW S.E. Coexistence among Epiphytic Bacterial populatins mediated through nutritional resource partitioning. *Aplied and Enviromental Microbiology*, **60** (12), 4468, **1994**.
14. WACHOWSKA U. Effect of Amistar 250SC and Bravo 500SC fungicides on the microorganisms colonising the winter wheat phyllosphere.(In Polish) *Advances in Plant Protection.* **39** (2), 882, **1999**.
15. WACHOWSKA U. Microorganisms colonising the leaves of winter wheat protected with the immunity stimulator Bion 50WG and fungicide Impact Super 347SC. (In Polish) *Year Book of Agricultural Academy in Poznań CCCXXI – Horticulture* **30**, 159, **2000**.

16. RIBEIRO I. C., VERISSIMA I., MONIZ L., CARDOSO H., SOUSA M.J., A.M.V.M. SOARES, LEAO C. Yeasts as a model for assessing the toxicity of the fungicides Penconazol, Cymoxanil and Dichlofluanid. *Chemosphere* **41**, 1637, **2000**.
17. HANAZATO T. Growth analysis of *Daphnia* early juvenile stages as an alternative method to test the chronic effect of chemicals. *Chemosphere* **36**, 1903, **1998**.
18. ROBINSON P. W. The toxicity of pesticides and organics to mysid shrimps can be predicted from *Daphnia* spp. Toxicity data. *Wat. Res.* **33**, (6), 1545, **1999**.
19. CABRAL M.G., VIEGAS C.A., TEIXEIRA M. C., SACORREIA I. Toxicity of chlorinated phenoxyacetic acid herbicide in the experimental eukaryotic model *Saccharomyces cerevisiae*: role of pH and of growth phase and size of the yeast cell population. *Chemosphere* **52**, 47, **2003**.
20. BARNETT H. L. Illustrated Genera of Imperfekt Fungi. Department of Plant Pathology, Bacteriology and Entomology. West Virginia University; pp. 225, **1965**.
21. BOOTH C. The Genus *Fusarium*. Commonwealth Mycological Institute Kew, Surrey, England; pp. 237, **1971**.
22. DOMSCH K. H., GAMS W. A manual of soil fungi. The Iowa State College Pres-Ames, Iowa, USA; pp. 440, **1972**.
23. ELLIS M.B. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute Kew, Surrey, England; pp. 597, **1971**.
24. FASSATIOVA O. Microscopic fungi in technical microbiology. (In Polish) WNT Warszawa; pp. 115, **1983**.
25. GAMS W. *Cephalosporium* – artige Schimmelpilze (*Hyphomycetes*). VEB Gustav Fischer Verlag. Jena; 253, **1971**.
26. GILMAN J.C. A manual of soil fungi. The Iowa State College Press-Ames, Iowa, USA; pp. 440, **1957**.
27. KWAŚNA H., CHEŁKOWSKI J., ZAJKOWSKI P. Fungi (Mycota). (In Polish) The Institute of Botany in Kraków; pp. 127, **1991**.
28. RIFAI M.A. A Revision of the genus *Trichoderma*. *Mycological Papers* **116**, 1, **1969**.
29. BURGIEL Z., GRABOWSKI M. The effect of selected herbicides on pathogenicity and growth of *Nectria galligena* Bres. *Folia Horticulturae, Ann.* **8/2**, 13, **1996**.
30. BARNETT J.A., PAYNE R.W. YARROW D. Yeasts: characteristics and identification. Cambridge University. pp. 1138, **2000**.
31. KLUCZEK J.P. Biochemical techniques of microorganism identification. (In Polish) ATR Bydgoszcz; pp. 95, **1999**.
32. KREGER – VAN RIJ N.J.W. The Yeasts: A Taxonomic Study. Elsevier Science Publishes Amsterdam; pp. 263, **1984**.
33. GULLINO M. L., LEROUX P. SMITH C. M. Use and challenges of novel compounds for plant disease control. *Crop Protection* **19**, 1, **2000**.
34. KURNATOWSKA A. Reservoirs of pathogenic biotic factors in the aerosphere, hydrosphere and lithosphere. In: Ecology and its connections with various sciences. (In Polish) Published PWN, pp. 215, **2002**.
35. DYNOWSKA M. Yeast and yeast-like fungi as pathogenic factors and bioindicators in water systems. (In Polish) WSP, Olsztyn, **1995**.