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THE EFFECT OF CULTIVATION CONDITIONS ON GROWTH, SPORULATION AND FORMATION OF MORPHOLOGICAL STRUCTURES OF *TOPOSPORA MYRTILLI* (FELTG.) BOEREMA

Mariusz Szmagara[1], Ewa Zalewska[2]

[1]Institute of Ornamental Plants and Architecture of Landscape, University of Life Sciences in Lublin, Leszczyńskiego 58
20-068 Lublin, Poland
e-mail: mariusz.szmagara@up.lublin.pl
[2]Department of Plant Pathology, University of Life Sciences in Lublin, Leszczyńskiego 7, 20-069 Lublin, Poland
e-mail: ewa.zalewska@up.lublin.pl

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Summary

The growth of *Topospora myrtilli* isolates, obtained in the years 2001-2003, was studied on PDA medium at –10, +2, +6, +12, +18, +22 and +28°C. The growth and sporulation of the isolates were observed at +22°C on the following culture media: PDA, maltose – MA and mineral – MSA, as well as on two versions of oat media, one containing 20 g of oat flakes per litre (OA-20), and the other containing 50 g of oat flakes per litre (OA-50). The observations of the linear growth of colonies of the studied strains were carried out during a period of 21 days, whereas the formation of morphological structures until the 52nd day of cultivation. It was found that *T. myrtilli* can grow and sporulate in a wide range of temperature, even at +2°C. The pathogen growth activates with the increase of temperature, up to the optimal temperature, i.e. from +18°C to +22°C. The increase of temperature to +28°C did not favour the mycelium growth and prevents pathogen sporulation. PDA, OA-20, OA-50 and MA media can be considered to be the most suitable for the growth and development of *T. myrtilli*, due to the intensive growth and formation of typical macro- and microscopic features. It is necessary to emphasise the great usefulness of oat medium, especially OA-50, for the cultivation of *Topospora myrtilli*, in the aspect of sporulation of the fungus. It was found that mineral medium is unsuitable due to the formation of mycelium with an untypical structure and colouration as well as late conidial sporulation.

Key words: *Topospora myrtilli*, temperature, culture medium, growth, sporulation

INTRODUCTION

The fungus *Topospora myrtilli* occurs on aboveground organs of ericaceous plants and it is particularly harmful to highbush blueberry (*Vaccinium corymbosum* L.) in all regions where this plant is cultivated (Weingartner and Klos, 1975; Rossman et al. 1987; Oudemans et al. 1998; Farr et al. 1995; Stromeng and Stensvand, 2001). In the years 2001-2003, the colonisation of aboveground organs of highbush blueberry by different fungi species was found (Machowicz-Stefaniak et al., 2002; Szmagara and Machowicz-Stefaniak 2005; Szmagara, 2006). One of them was *T. myrtilli,* frequently isolated from necrotic, ellipsoidal, brown chestnut-coloured spots. Canker spots caused by this pathogen were primarily located at the shoot base, but also in the higher portions, most often around leaf marks and buds. Pycnidia arranged in concentric circles frequently occurred on the surface of these spots, and the tissue in the central part was grey-ashen in colour (Weingartner and Klos, 1975; Borecki and Pliszka, 1978; Stromeng and Stensvand, 2001; Szmagara and Machowicz-Stefaniak, 2005; Szmagara, 2006). The microscopic examination of the pycnidia showed that there were in them numerous conidial spores typical for the genus *Topospora* (Sutton, 1980). The susceptibility of highbush blueberry shrubs to *T. myrtilli* is the greatest in the autumn and spring periods (Borecki and Pliszka, 1978; Stromeng and Stensvand, 2001). It suggests that both temperature and air relative humidity are factors which affect the pathogen development.

Given the absence of information on life requirements of *Topospora myrtilli*, studies were undertaken in order to determine growth conditions, sporulation and different macro- and microscopic features of the fungus.

MATERIALS AND METHODS

The studies on growth and sporulation, included three single spore isolates of *Topospora myrtilli*: Bw 77, Bw 121 and Bw 122, isolated from highbush blueberry shoots in the years 2001-2003. Each of them was cultured at the following temperatures: -10, +2, +6, +12, +18, +22, +28°C, on PDA medium (Difco). In the studies on growth and sporulation of *T. myrtilli* on five culture media: potato – PDA, maltose – MA, mineral – MSA, as well as two oat media: with the content of 20g of oat flakes per 1 litre – OA 20, and 50g of oat flakes per 1 litre – OA 50 (Zalewska and Machowicz-Stefaniak, 2000; www.be.cabri.org; www.ifo.or.jp/index_e.html), the cultures of six pathogen isolates were investigated, i.e.: Bw 1535, Bw 1577, Bw 2462, Bw 2934, Bw 3133, Bw 3135. The cultures were grown at a temperature of 22°C, on solidified agar media on Petri dishes on which the inocula of the studied fungus were placed. The inoculation material comprised 3 mm-diameter discs excised from seven-day-old mother colonies which were grown in dispersed light on PDA medium at a temperature of 22°C (Zalewska and Machowicz-Stefaniak, 2000). For each isolate and temperature, 4 replicates were used, treating each dish as a replicate.

The observations of the linear growth of colonies of the studied strains were carried out during a period of 21 days, whereas the formation of morphological structures until the 52nd day of cultivation. The colony diameter was measured every second day, making two crosswise measurements for each replicate. At the same time, the appearance of the colony was observed, as well as the formation of pycnidia and conidial spores of the pathogen was checked under the microscope. The formation of morphological structures of *T. myrtilli* was examined by light microscopy and scanning electron microscopy (SEM). The obtained data were subjected to statistical analysis using variance analysis and Tukey's confidence intervals.

RESULTS

As a result of the conducted experiment it was found that there were significant differences in the growth of *T. myrtilli* colonies, depending on the culture temperature (Tab. 1). The studied fungus' isolates on PDA medium at a temperature of -10°C did not produce aerial mycelium until the 21st day of cultivation (Tab. 1). Having transferred the inoculum to the dishes at 22°C, a slight aerial mycelium developed around the inoculum after 2 days of culturing, and after subsequent days the linear growth of the colony was observed.

A small growth of the colony was observed in the isolates stored at 2 and 6°C after 4 and 6 days (Fig. 1). After 14 days the colony diameter of the isolates grown at a temperature of 2°C did not exceed 10 mm, and at 6°C about 20 mm (Tab. 1, Fig. 1). The pathogen colonies increased quite slowly at a temperature of 12 and 28°C, in spite of the fact that their growth started already after 2 days of incubation. The growth of *T. myrtilli* at a temperature of 18 and 22°C also started after 2 days of culturing. After 10 days the colony diameter already exceeded 40 mm, and after 14 days it was between about 53 mm and 62 mm at a temperature of 18°C, and between 52.5 mm and 58.8 mm at 22°C (Fig. 1). The above-mentioned values are significantly larger than the colony diameters of the studied isolates cultured at the following temperatures: -10, 2, 6, 12 and 28°C (Tab. 1). Only in the case of the strains Bw 77 and Bw 121, the diameter of 14-day-old colonies cultured at 18°C was significantly larger than that of the isolate Bw 122 grown in the same conditions. In the case of other temperature values, no significant differences were found in the colony diameters of particular isolates (Tab. 1).

It was observed that the colonies of *T. myrtilli* grown at 22°C for 21 days produced single, flask-shaped or globose pycnidia, primarily located in the central part of the colony (Fig. 2). Inside the pycnidia, there were numerous conidial spores typical for this species (Fig. 3). At other temperature values, the pathogen colonies did not produce pycnidia until the 21st day of cultivation.

The studies of the effect of culture medium type on the growth and sporulation of *T. myrtilli* at 22°C showed that 14-day-old colonies of the studied isolates on mineral medium had the smallest diameter ranging between about 29 and 46.1 mm (Tab. 2, Fig. 4). The largest diameter was noted in the isolates Bw 1535 and Bw 2462 on PDA medium and it was, respectively, 64.7 mm and 64.2 mm. After the same time, the colony diameter of particular isolates on maltose medium was from about 50 to 66 mm. On OA-20 and OA-50 medium, the colony diameter ranged between about 55 and 62 mm (Tab. 2, Fig. 4).

The statistical analysis of the obtained results showed that the size of 14-day-old colonies of all the isolates grown on mineral medium was significantly smaller than the size of the colonies of these isolates cultured on the other culture media. But the size of the fungus colony on OA-20, OA-50 medium and maltose medium, except for the isolate Bw 3135, did not differ significantly. The diameter of the *T. myrtilli* colony on PDA medium was significantly larger than that on maltose medium only in the case of the isolate Bw 2934, and significantly smaller in the case of the isolate Bw 1577 (Tab. 2, Fig. 4). No significant differences were found in the size of 14-day-old colonies of the studied isolates grown on a given culture medium (Tab. 2).

Table 1
The effect of temperature on the diameter of colonies of *T. myrtilli* (mm) after 14 days of growth on PDA medium.

Isolate no. \ Temperature [°C]	-10	2	6	12	18	22	28
Bw 77	no growth	5.78 a A	16.75 b A	39.25 c A	61.75 d A	58.75 d A	11.75 ab A
Bw 121	no growth	9.87 a A	20.37 b A	40.50 c A	61.50 d A	53.25 e A	13.63 ab A
Bw 122	no growth	8.37 a A	18.25 b A	34.75 c A	53.00 d B	52.50 d A	9.87 a A
$NIR_{0,01}$ $LSD_{0.01}$				7.95			

The means differ significantly (P≤ 0,01) if they are not marked with the same letter
small letters – differences depending on temperature for a given isolate
capital letters – differences between isolates at a given temperature

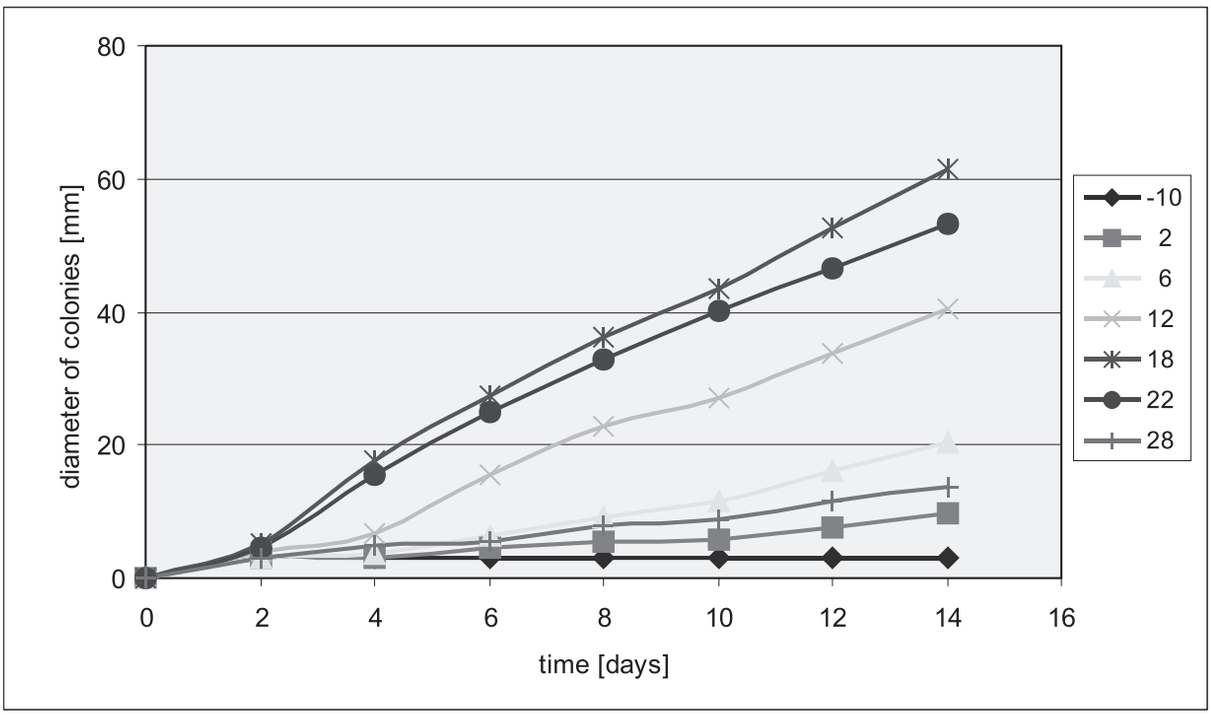

Fig. 1. The growth of colonies of *Topospora myrtilli* isolate Bw 121 at various temperature on PDA medium.

Table 2
The effect of culture media on the diameter (mm) of 14-day-old colonies of *Topospora myrtilli* at 22°C.

Isolate no. \ Culture media	potato PDA	maltose MA	mineral MSA	oat OA-20	oat OA-50
Bw 1535	64.7 gh C	57.6 d-h B	29.3 a A	62.0 f-h B	61.6 f-h B
Bw 1577	51.8 c-e C	63.8 f-h B	46.1 bc A	54.8 c-f B	57.6 d-h B
Bw 2462	64.2 f-h C	56.9 d-h B	36.8 ab A	56.3 d-h B	55.6 c-g B
Bw 2934	60.5 e-h C	50.2 cd B	32.8 a A	57.0 d-h B	56.9 d-h B
Bw 3133	63.9 f-h C	55.1 c-g B	30.5 a A	54.6 c-f B	54.7 c-f B
Bw 3135	61.9 f-h C	65.7 h B	37.1 ab A	56.1 d-h B	55.5 c-g B

The means marked with the same letter do not differ significantly;
small letters – differences depending on culture medium for a given isolate $LSD_{0,05} = 9.74$
capital letters – differences between isolates on a given culture medium $LSD_{0,05} = 2.86$

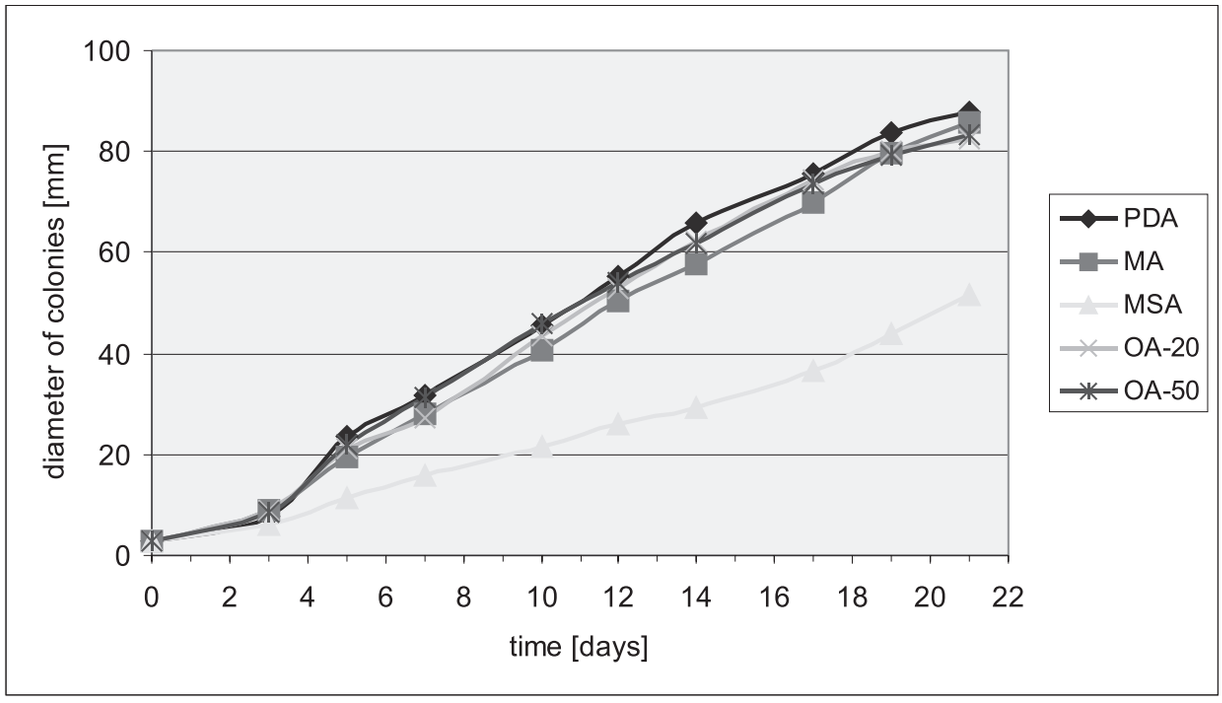

Fig. 4. The growth of colonies of *T. myrtilli* isolate Bw 1535 on different media at 22°C.

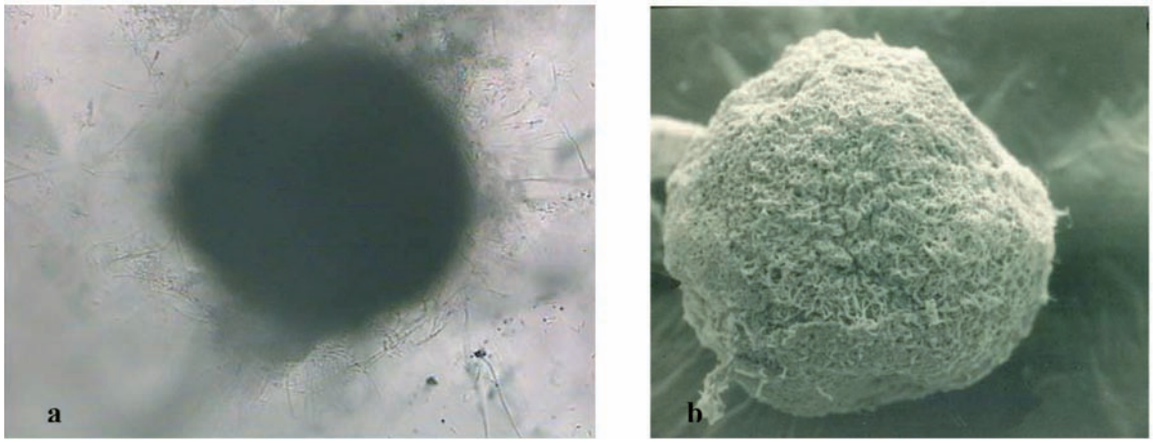

Fig. 2. *T. myrtilli:* a) pycnidium with visible ostiole 120x (photo by M. Szmagara), b) pycnidium, SEM, 175x (photo by M. Szmagara, M. Wróbel).

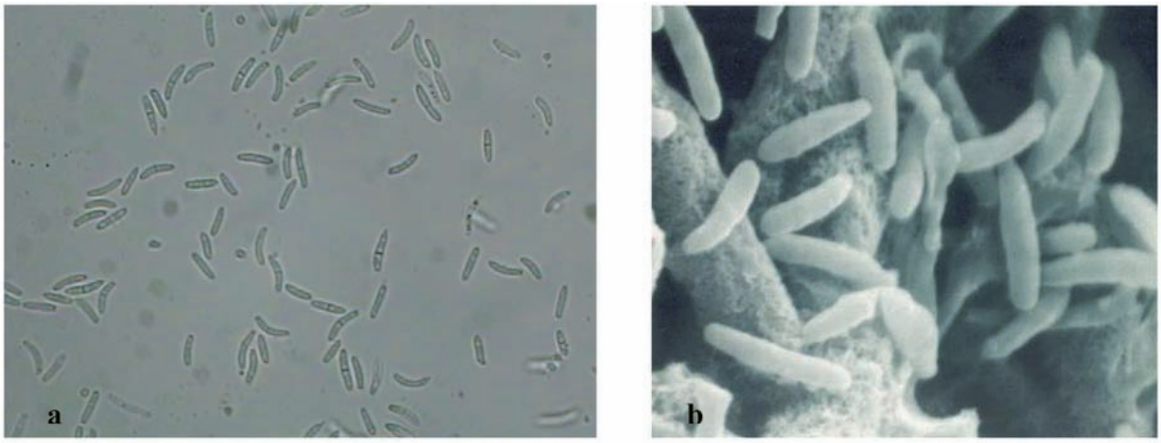

Fig. 3. Conidia of *T. myrtilli:* a) 750x (photo by M. Szmagara), b) SEM, 6429x (photo by M. Szmagara, M. Wróbel).

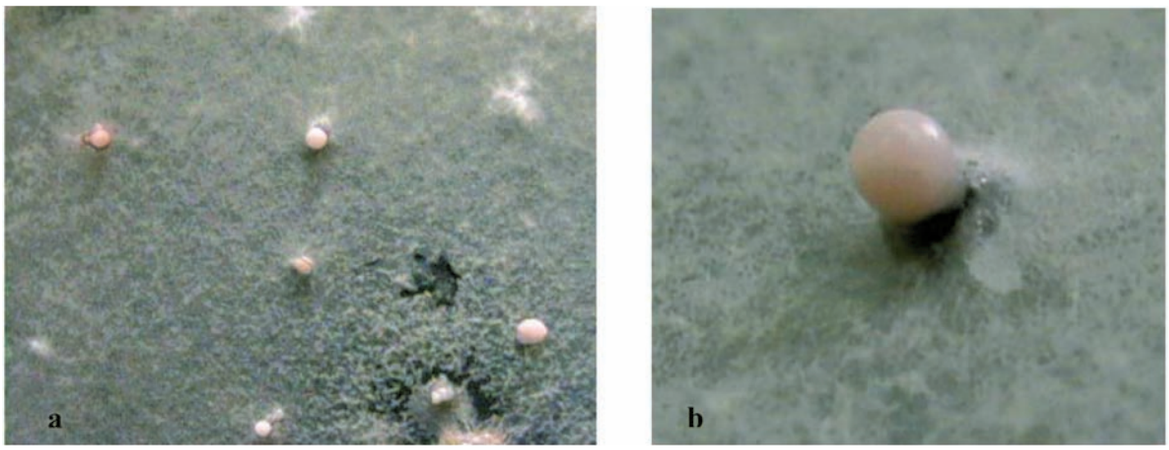

Fig. 5. Exudate from pycnidium of *T. myrtilli* isolate Bw 1535 on OA-20 medium on 42nd day of cultivation: a) 2x, b) 4x (photo by M. Szmagara).

The rate of linear growth of particular fungus isolates on the investigated media was similar (Fig. 4).

The conducted studies showed large differences in macroscopic and microscopic features of the studied isolates of *T. myrtilli*, depending on culture medium. The colonies were bright lemon-green on PDA medium, green-grey on maltose medium, and grey-green on OA-20 and OA-50 medium. The intensive yellow colour of the colony was observed in all the isolates on mineral medium.

The colony structure was most frequently velvety flocculent or felt-like, and on mineral medium it was cotton wool-like.

The colour of the reverse side on PDA and maltose medium was dark green-brown, gradually brightening to the white colour towards the edge of the colony. On OA-20 and OA-50 medium, it was bottle green in colour, and on mineral medium - dark green turning into yellow-green.

The formation of pycnidia was observed earliest on OA-50 medium, as early as on the 19th day of culturing (Fig. 2). They were concentrated in three sectors. Inside the pycnidia, there were conidial spores typical for this species (Fig. 3). On OA-20, maltose and PDA medium, the pycnidia and conidia formed on the whole surface, with their high concentration in the central part of the colony. The first pycnidia were noticed on the 21st day of growth, and after 28 days they were very numerous. On mineral medium, certain isolates formed sclerotia around the inoculum, from strongly dense, short mycelial hyphae. The formation of pycnidia was noted only after 42 days of culturing. They were gathered in aggregates and filled with conidial spores.

The pycnidia of *T. myrtilli* were well visible, in particular on PDA medium, in the form of dark spots. They formed mainly in the central part of the colony and were embedded in the substrate mycelium or slightly protruded above the surface (Fig. 2). After four weeks of cultivation, the formation of aggregates of pycnidia was observed. After 19 or 42 days, depending on the culture medium, salmon-orange thick drops came out of the ostioles of the pycnidia in which there were numerous spores of the pathogen (Fig. 5). The conidia were typical for the species, most frequently with one septum, two-chambered, colourless, straight, curved, fusiform or ellipsoidal (Fig. 3). Their dimensions were similar on particular culture media and within the range given by Sutton (1980).

DISCUSSION

The presently demonstrated ability of mycelium development in the studied isolates of *T. myrtilli* in a wide range of temperatures probably enables the colonisation of plant shoots in moderate climate conditions (Borecki and Pliszka, 1978; Stromeng and Stensvand, 2001). It also indicates the possibility of vegetative growth of the fungus even at a temperature of +2ºC. With a temperature increase, the growth of the pathogen becomes more active, up to the optimal temperature within the range from +18 to 22ºC. The temperature of +22ºC proved to be optimal for conidial sporulation of *T. myrtilli*. In the light of the obtained results, the temperature of +28ºC does not promote mycelium development and prevents pathogen sporulation. Thus, it may be suggested that hot days are not favourable for the development of canker of highbush blueberry shoots caused by *T. myrtilli*. In spite of the absence of growth of mycelium of the pathogen at -10ºC, it was found that it does not lose its living abilities, but regenerates with a progressive increase of temperature. It means that a temperature below -10ºC is lethal for hyphae of the pathogen. Such properties of the mycelium explain the pathogen's ability to winter over on perennial shoots, in particular in their lower portions (Borecki and Pliszka, 1978; Borecki, 1990; Bielenin, 1997; Stromeng and Stensvand, 2001).

The conducted studies showed that, due to the intensive growth and the development of typical macro- and microscopic features of the studied isolates, the following media should be considered to be the most suitable for the growth and development of *T. myrtilli*: PDA medium, OA-20 and OA-50 oat medium, as well as maltose medium. Among the above-mentioned media, we should emphasise the great usefulness of oat medium, especially OA-50, for the cultivation of *Topospora myrtilli*, in the aspect of sporulation of the fungus. Likewise, in the case of *Phoma* spp., *Seimatosporium hypericinum* and *Phomopsis viticola*, oat medium favours the growth and sporulation of the abovementioned fungi, hence its frequent use for species identification (Boerema, 1976; Gruyter and Noordeloos, 1992; Coęlho et al. 1997; Zimowska, 2002).

Mineral medium, due to the formation of mycelium with an untypical structure and colouration as well as late conidial sporulation, should be considered unsuitable for the cultivation of *T. myrtilli*, in spite of its common use for isolation of many other fungi species from plant tissues (Łacicowa, 1976).

The usefulness of organic media for the cultivation of this fungus and its weak growth on mineral medium is also stressed by Borecki and Pliszka (1978), and in the case of pathogens isolated from shoots of other orchard plants, e.g. hazel and grape vine, by Kuropatwa (1993), Machowicz-Stefaniak and Zalewska (2000).

CONCLUSIONS

1. The development of the mycelium of *T. myrtilli* within the temperature range from 2 to 22ºC probably enables the pathogen to colonise highbush blueberry shoots in moderate climate conditions.
2. The temperature from 18 to 22ºC proved to be optimal for the growth, and 22ºC for the production of conidial spores.
3. The lethal temperature for the growth of the mycelium of *T. myrtilli* is the temperature below -10ºC.
4. Maltose medium proved to be the most suitable for isolation of *T. myrtilli*, whereas PDA, OA-20 and OA-50 medium for sporulation.

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http://www.be.cabri.org/ on line CABRI – Common Access to Biological Resources and Information

http://www.ifo.or.jp/index_e.html on line IFO – Institute for Fermentation, Osaka

Wpływ warunków hodowli na wzrost, zarodnikowanie i tworzenie struktur morfologicznych *Topospora myrtilli* (Feltg.) Boerema

S t r e s z c z e n i e

Wzrost izolatów *Topospora myrtilli,* uzyskanych w latach 2001-2003 badano w temperaturze: – 10, +2, +6, +12, +18, +22 i +28ºC na pożywce PDA. Ponadto w temperaturze +22ºC obserwowano wzrost i zarodnikowanie izolatów na pożywkach: PDA, MA, MSA oraz dwóch pożywkach owsianych: jednej o zawartości 20 g płatków owsianych na 1 litr – OA – 20, drugiej 50 g płatków owsianych na 1 litr – OA – 50. Obserwacje wzrostu liniowego kolonii badanych szczepów prowadzono przez 21 dni, a tworzenie struktur morfologicznych do 52-ego dnia hodowli. Ustalono, że *T. myrtilli* może rosnąć i zarodnikować w szerokim zakresie temperatury. Wskazuje na to możliwość wzrostu wegetatywnego grzyba nawet w temperaturze +2ºC. Wraz ze wzrostem temperatury uaktywnia się rozwój patogena, aż do temperatury optymalnej od +18ºC do +22ºC. Temperatura +28ºC nie sprzyja rozwojowi grzybni i uniemożliwia zarodnikowanie patogena. Ze względu na intensywny wzrost i tworzenie typowych cech makro- i mikroskopowych za najodpowiedniejsze podłoża dla wzrostu i rozwoju *T. myrtilli* można uznać pożywki: PDA, owsianą OA-20, owsianą OA-50 oraz maltozową. Spośród wymienionych należy podkreślić dużą przydatność do hodowli *T. myrtilli* pożywki owsianej, a zwłaszcza OA-50 w aspekcie zarodnikowania grzyba. Pożywka mineralna okazała się nieodpowiednia, ze względu na tworzenie grzybni o nietypowej strukturze i zabarwieniu oraz późne zarodnikowanie konidialne.