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IN VITRO ANTIFUNGAL AND DEMELANIZING ACTIVITY OF NEPETA RTANJENSIS ESSENTIAL OIL AGAINST THE HUMAN PATHOGEN *BIPOLARIS SPICIFERA*

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Abstract – The antifungal activity of *Nepeta rtanjensis* Diklić & Milojević essential oil was tested against the human pathogenic fungus *Bipolaris spicifera* (Bainier) Subramanian via mycelial growth assay and conidia germination assay. The minimally inhibitory concentration (MIC) of the oil was determined at 1.0 μg ml^{-1}, while the MIC for the antifungal drug Bifonazole in a positive control was determined at 10.0 μg ml^{-1}. The maximum of conidia germination inhibition was accomplished at 0.6 μg ml^{-1}. In addition, at 0.6 μg ml^{-1} and 0.8 μg ml^{-1} the oil was able to cause morphophysiological changes in *B. spicifera*. The most significant result is the bleaching effect of the melanized conidial apparatus of the test fungi, since the melanin is the virulence factor in human pathogenic fungi. These results showed the strong antifungal properties of *N. rtanjensis* essential oil, supporting its possible rational use as an alternative source of new antifungal compounds.

Key words: Bipolaris spicifera (Bainier) Subram., essential oil, antifungal activity, melanin, human pathogen, depigmentation

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INTRODUCTION

Bipolaris spicifera (Bainier) Subram. (telemorph: *Cochliobolus spicifer* R.R. Nelson) is dematiaceous hyphomycete very often isolated from plant material, which in regions with a hot and dry climate are among the most frequent air-borne fungi encountered (Alcorn, 1988). *B. spicifera* appears frequently in medical literature as a cause of human and animal diseases: cutaneous and subcutaneous phaeohyphomycosis (Mc Ginnis et al., 1992), fungal sinusitis (Buzina et al., 2003), cutaneous disease (Straka et al., 1989), mycotic keratitis (Hemashettar et al., 1992; Saha and Das 2005), allergic fungal sinusitis (Taguchi et al., 2004), systemic dual mycosis with *Torulopsis glabrata* in a dog (Waurzyniak et al., 1992), fatal endarteritis after aortic valve replacement (Ogden et al., 1992), fungal peritonitis (Bava et al., 2003), meningitis (Latham, 2000) and disseminated disease in a neonate (Moore et al., 2001). Dematiaceous fungi, including *Bipolaris* species are the most common agents involved in allergic fungal sinusitis (Castelnuovo et al., 2003). Although *B. spicifera* infections are not frequent they must not be neglected. Dematiceous fungi are characterized by the presence of dark brown pigment – melanin within their cell wall structure. Melanins are negatively charged, hydrophobic biopolymers with high molecular weights, typically brown or black, formed by the oxidative polymerization of phenolic or indolic compounds, by organisms in all biological kingdoms, including fungi. Fungal melanins are usually found in the cell walls of spores, sclerotia, mycelia or fruiting bodies (Butler and Day 1998). They enable fungi to survive adverse environmental conditions by protecting them against oxygen free radicals (Romero-Martinez et al., 2000), UV radiation (Kawamura et al., 1997), and wall-degrading enzymes produced by antagonist microbes (Butler et al., 2001). Melanins also have genoprotective effects (Babitskaya et al., 2000). Many human pathogenic

fungi contain melanin within their cell wall structure (e.g. *Aspergillus fumigatus*, *A. nidulans*, *A. niger*, *Alternaria alternata*, *Cladosporium carionii*, *Cryptococcus neoformans*, *Exophiala jeanselmei*, *Fonsecaea compacta*, *Fonsecaea pedrosoi*, *Hendersonula toruloidii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Penicillium marneffei*, *Phaeoannellomyces wernickii*, *Phialophora richardsiae*, *P. verrucosu*, *Sporothrix schenckii*, *Wangiella dermatitidis*). For several of these fungi, melanin has been described as a virulence factor due to its ability to reduce a pathogen's susceptibility for killing by host antimicrobial mechanisms and by influencing the host immune response (Youngchim et al., 2004; Nosanchuk and Casadevall, 2006). Due to protective role of fungal melanin, dematiceous fungi are extremely difficult to treat with antifungal drugs (Nosanchuk and Casadevall, 2006). Plant secondary metabolites could be a good alternative for the treatment of fungal infections in light of increasing fungal resistance to commercial antifungal agents (Vivek et al., 2009). It has been shown that the essential oil isolated from *Nepeta rtanjensis* Diklić & Milojević (Lamiaceae), an endemic and critically endangered aromatic plant from south-east Serbia, has a strong antifungal activity and can inhibit the mycelial growth of some fungi *in vitro* (Stojanović et al., 2005; Ljaljević Grbić et al., 2008). The main components of the *N. rtanjensis* essential oil include α-pinene (3.3%), β-pinene (0.37%), 2-metoxy-p-cresol (1.14%), 4aβ,7α,7aβ nepetalactone (6.30%), α-copaene (1.33%), 4aα,7α,7aβ nepetalactone (79.89%), germacrene D (1.80), δ-cadinene (2.12%) (Ljaljević Grbić et al., 2008).

The present research emphasizes the antifungal activity of *N. rtanjensis* essential oil against *B. spicifera* and its potential to cause the demelanizing (bleaching) of *B. spicifera* reproductive structures.

MATERIAL AND METHODS

Essential oil

The essential oil was isolated from air-dried aerial parts of *Nepeta rtanjensis*, collected during the pre-flowering stage, by hydrodistilation for 2 h in a Clavenger-type apparatus. The extracted essential oil was kept in sealed glass vials at + 4˚C until further analysis.

Fungal strain used

Bipolaris spicifera (Bainier) Subram. was originally isolated from the wall of a storage room of the Serbian National Museum. Due to the high concentration of indoor air fungal spores, the room suffered from "sick building syndrome". The fungus was deposited to the Mycotheca of the Department of Algology, Mycology and Lychenology, Faculty of Biology, University of Belgrade. The fungus was maintained on a malt extract agar (MEA), and potato dextrose agar (PDA), stored at + 4˚C and subcultured once in a month.

Test for antifungal activity

Mycelial growth assay

Different concentrations of essential oil (0.2 – 1.4 μg ml^{-1}) were diluted in Petri dishes with 10 ml of MEA. For each treatment and each dose tested, three replicate Petri dishes were used. The culture medium was inoculated with 5 mm agar discs from an actively growing culture of *B. spicifera*. After 21 days of incubation in the dark at + 25˚C, the diameter of the colonies was recorded. Antifungal activity was expressed in terms of percentage of mycelia growth inhibition and calculated using the formula of Pandey et al., 1982):

growth inhibition %= 100 (dc – dt)/dc

dc = average diameter of fungal colony in control

dt = average diameter of fungal colony in treatment.

Petri plates with the commercial fungicide, Bifonazole, were used as a positive control. The experiments were repeated twice. The minimum inhibitory concentration (MIC) of oil necessary for the inhibition of mycelia growth of the fungal strain was determined by the method described by Ishii (1995).

Conidia germination assay

Conidia germination assays were carried out on Petri dishes containing MEA amended with different *N. rtanjensis* essential oil concentrations (0.2 – 1.4 μg ml^{-1}). A MEA without essential oil was used as a negative control. For each treatment and each dose tested, three replicate Petri dishes were used. Petri dishes were inoculated by covering the entire surface with a suspension of 200 μl of *B. spicifera* conidia (10^4 ml^{-1}), obtained from the sporulated mycelia of 10-day-old cultures, and incubated in the dark at +25˚ C. After 24 h, germinated and non-germinated conidia were counted under a microscope (Zeiss Axio Imager M.1, with AxioVision Release 4.6 software). At least 200 conidia were counted for each observation and scored by hemocytometer. Conidia were considered germinated when the germ tube length was at least half the length of the diameter of the conidia or longer. The experiments were repeated twice.

Screening of morphophysiological changes

Light microscopy

A sample of mycelium was taken from the periphery of a colony grown on MEA enriched with different concentrations of *N. rtanjensis* essential oil. The samples were dyed and fixed with lactophenol – cotton blue and observed under a light microscope (Zeiss Axio Imager M.1, with AxioVision Release 4.6 software) to examine structural abnormalities. Samples from the control plate without oil were also stained and observed.

Scanning electron microscopy (SEM)

Treated and control *B. spicifera* colonies were used for SEM observations. 5 x 10 mm segments were cut from the culture growing on the MEA and placed in vials containing 3% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) at 4˚C. Samples were kept in this solution for 48 h and then washed with distilled water and dehydrated in an ethanol. Then the samples were dried in liquid carbon dioxide and placed in desiccators until further use. The fungal materials were deposited on adhesive tape fixed to specimen tabs and then ion sputter coated with gold. Microstructure characterization of the samples was carried out with a JEOL JSM 6460 LV instrument equipped with an OXFORD INSTRUMENTS EDS analyzer.

Statistical analysis

One way ANOVA was performed for mycelial growth assay and conidia germination assay. A P value less than 0.05 was considered statistically significant.

RESULTS

Nepeta rtanjensis essential oil showed a strong antifungal and bleaching activity against mycelial growth and conidia germination of *Bipolaris spicifera* (Table 1). The radial growth of the fungal colony was sig-

Table 1. Inhibition of mycelial growth and conidia germination of *Bipolaris spicifera* in MA amended with different *Nepeta rtanjensis* essential oil doses:

Concentration of oil (μg ml^{-1})	Radial growth inhibition (%)	Conidia germination inhibition (%)	Depigmentation
0.2	1.93 ± 0.05	69.78 ± 0.37	-
0.4	7.07 ± 0.45	91.40 ± 1.92	-
0.6	38.98 ± 0.57	100	±
0.8	59.99 ±1.30	100	+
1.0	100	100	/
1.2	100	100	/
1.4	100	100	/

Each data point represents the mean value with standard error (P<0.05);
- melanized, + hyaline, ± partly melanized / no visible growth;

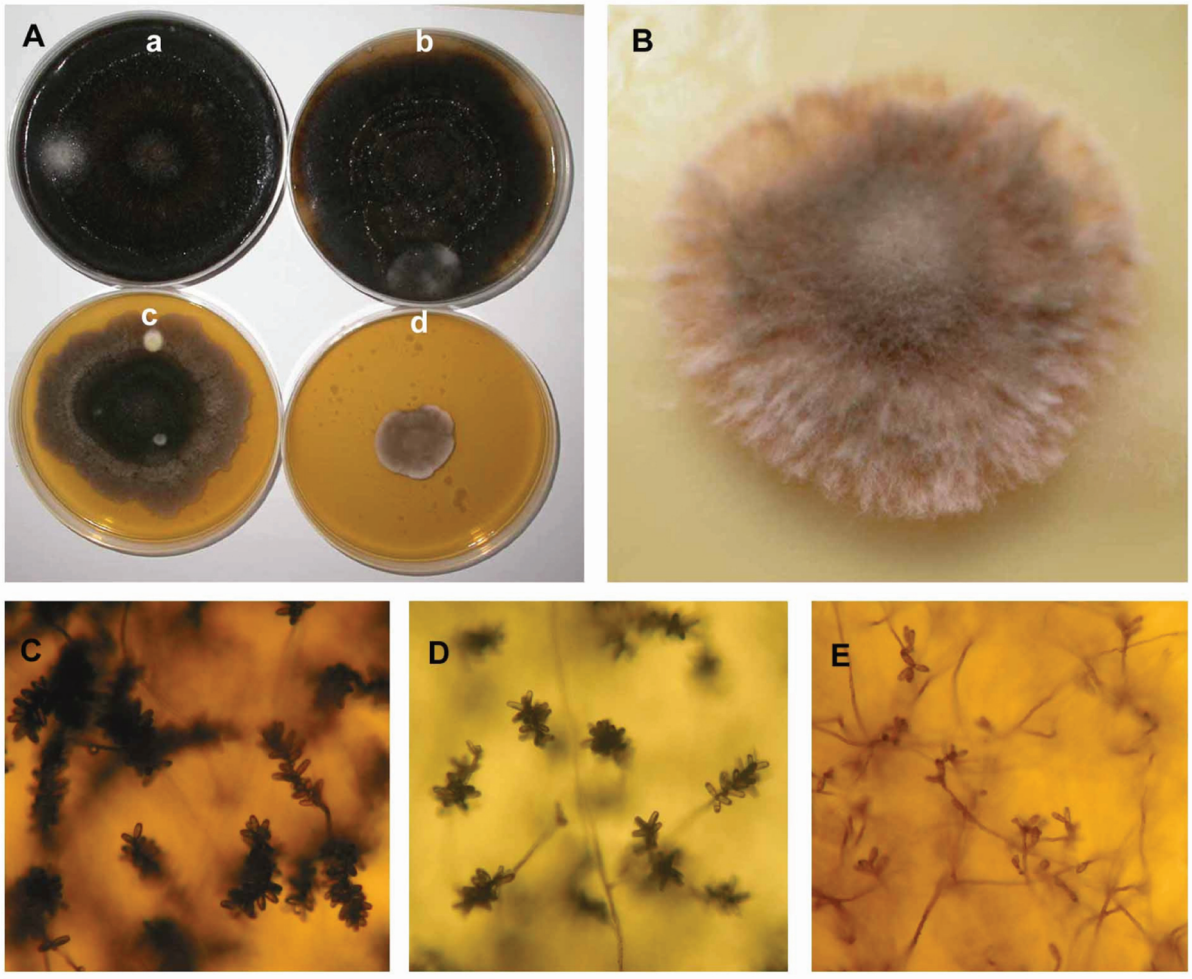

Fig. 1. The influence of *Nepeta rtanjensis* essential oil on *Bipolaris spicifera* mycelia growth. A. a) Negative control; b) Positive control; c) 0.6 μg ml^{-1}; d) 0.8 μg ml^{-1}; B. the detail of Ad; C. Normal conidia production in control culture; D. The lowest conidia production in culture with Bifonazole added; E. The pore conidia production in culture with 0.8 μg ml^{-1}essential oil added (C,D,E, 50x magnification).

nificantly reduced in response to different concentrations of essential oil. At 1.0 μg ml^{-1} the inhibition of fungal development reached its maximum (100% of radial growth inhibition) and this concentration was regarded as the MIC for the test fungus ($P<0.05$). The MIC for the commercial fungicide Bifonazole was determined at 10 μg ml^{-1}. In the conidia germination assay the maximum of conidia germination inhibition (100%) was accomplished at 0.6.μg ml^{-1} ($P<0.05$). In addition to inhibited mycelial growth and inhibition of conidia germination, the presence of different concentrations of essential oil seemed to exhibit distinct morphophysiological changes. These variations included lack of sporulation, visible loss of pigmentation and aberrant development of conidiophores. Colonies formed on the MEA enriched with 0.8 μg ml^{-1} essential oil were hyaline (Fig 1Ad). The intensity of sporulation was slightly reduced in the positive control (Fig 1D) and very poor on the MEA with essential oil added (Fig 1E). Light microphotographs showed changes in conidiogenous apparatus of *B. spicifera*. Typical micromorphology of *B. spicifera*, dark and pheoid septate hyphae, zig-zag conidiophores with thick walled cylindrical poroconidia appeared in the negative control (Fig. 2A). Low aberrant conidiophore, with only terminal zig-

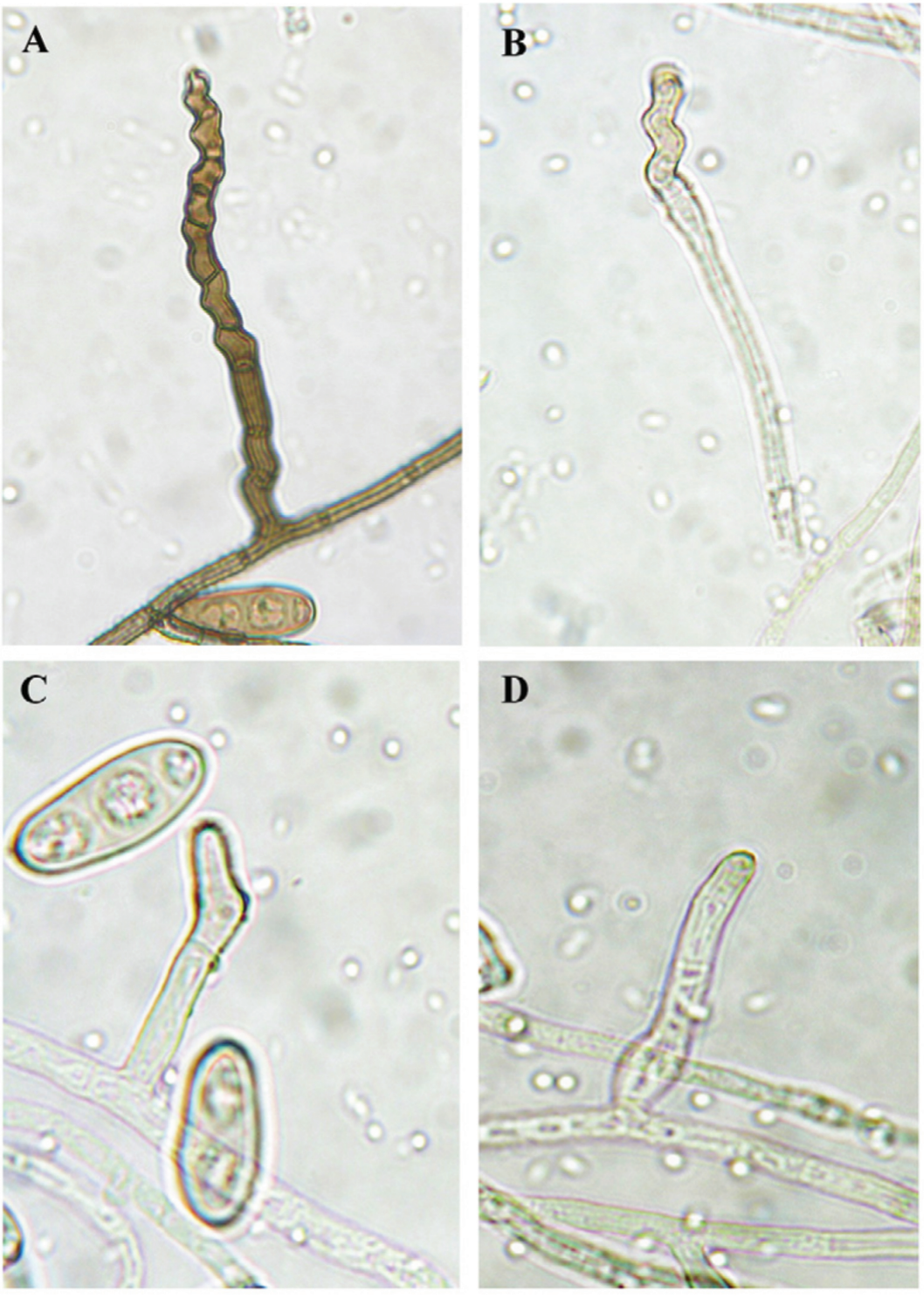

Fig. 2. Morphological changes of *Bipolaris spicifera* conidiogenous apparatus influenced by *Nepeta rtanjensis* essential oil (400x magnification): A. Negative control-typical zig-zag, melanized conidiophore; B. 0.6 μg ml^{-1} essential oil added-only terminal zig-zag appearance and slight melanization; C,D. 0.8 μg ml^{-1} essential oil added-short, conidiophores without zig-zag tips, bleached.

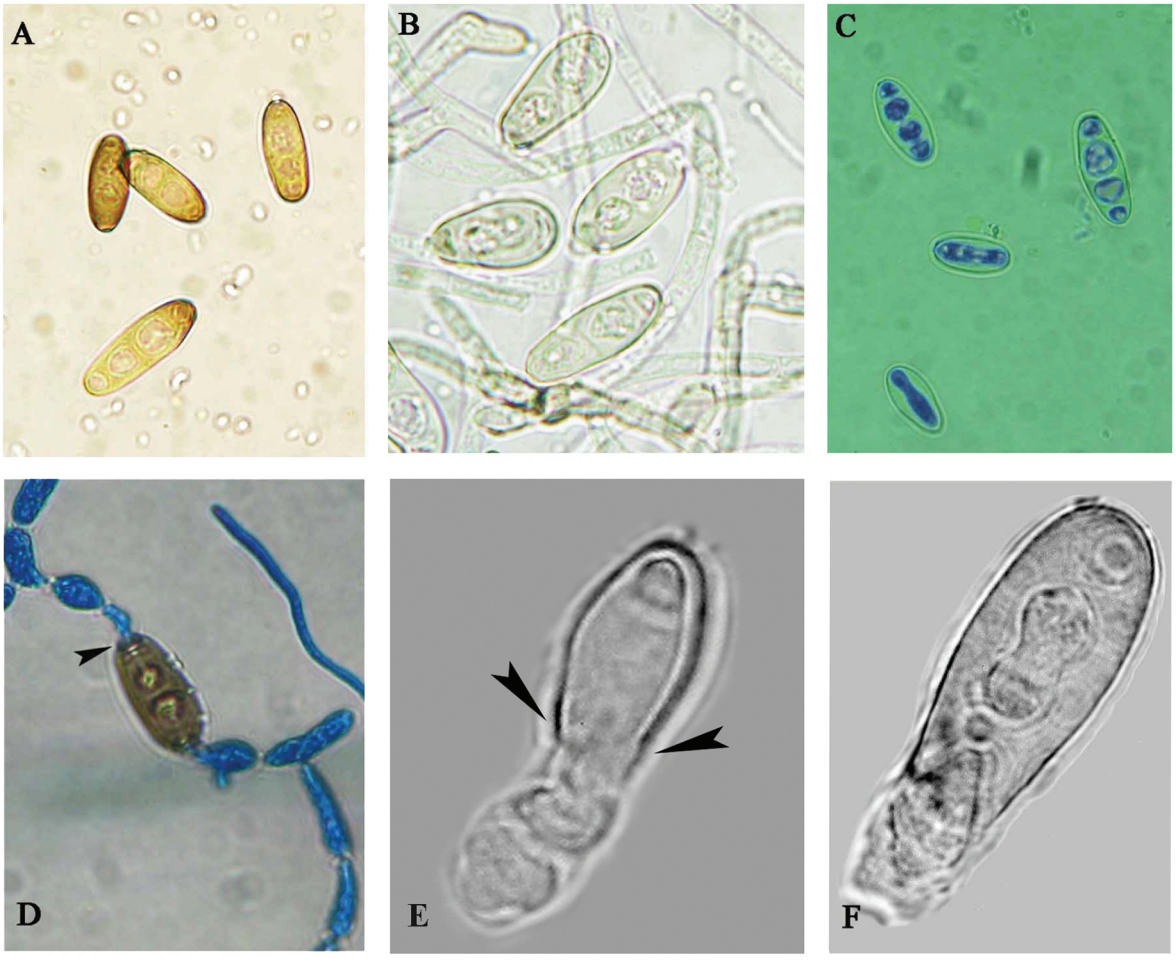

Fig. 3. Morphological changes of *Bipolaris spicifera* conidia influenced by *Nepeta rtanjensis* essential oil: A. Negative control-typical melanized poroconidia; B,C. Hyaline, bleached conidia-0.8 μg ml^{-1} essential oil added; D. Negative control-typical bipolar germination of poroconidia (arrow pointed terminal pore with germinating hyphae). E,F. Ungerminated, lysed conidia with cell wall rupture (arrow) -0.8 μg ml^{-1} essential oil added (A,B,C,D, 400x magnification; E,F, 1000x magnification).

zagging and slightly melanized, occurred on 0.6 μg ml^{-1} essential oil added (Fig. 2B). Aberrant conidiophores, non-zig-zagging, hyaline, non-melanized and hyaline poroconidia were recorded on 0.8 μg ml^{-1} essential oil added (Fig. 2C, D). The essential oil concentration at 0.8 μg ml^{-1} induced bleaching of the conidia (Fig.3B, C) and cell wall rupture (Fig.3D) with cell content flowing outside (Fig.3E). SEM microphotographs showed typical, roughed, zig-zag conidiophore with several poroconidia in the control (Fig.4D), in comparison to smooth sterile conidiophores or conidiophores with only terminal poroconidia (Fig. 4A, B). Cell wall rupture was also visible on the SEM microphotographs (Fig. 4C).

DISCUSSION

Nepeta rtanjensis essential oil showed the ability to interfere with all stages in the reproduction cycle of the human pathogenic fungus *Bipolaris spicifera*: conidia germination, mycelial growth and intensity of sporulation which is demonstrated with radial mycelial growth inhibition, inhibition of conidia germination and low conidia production in the

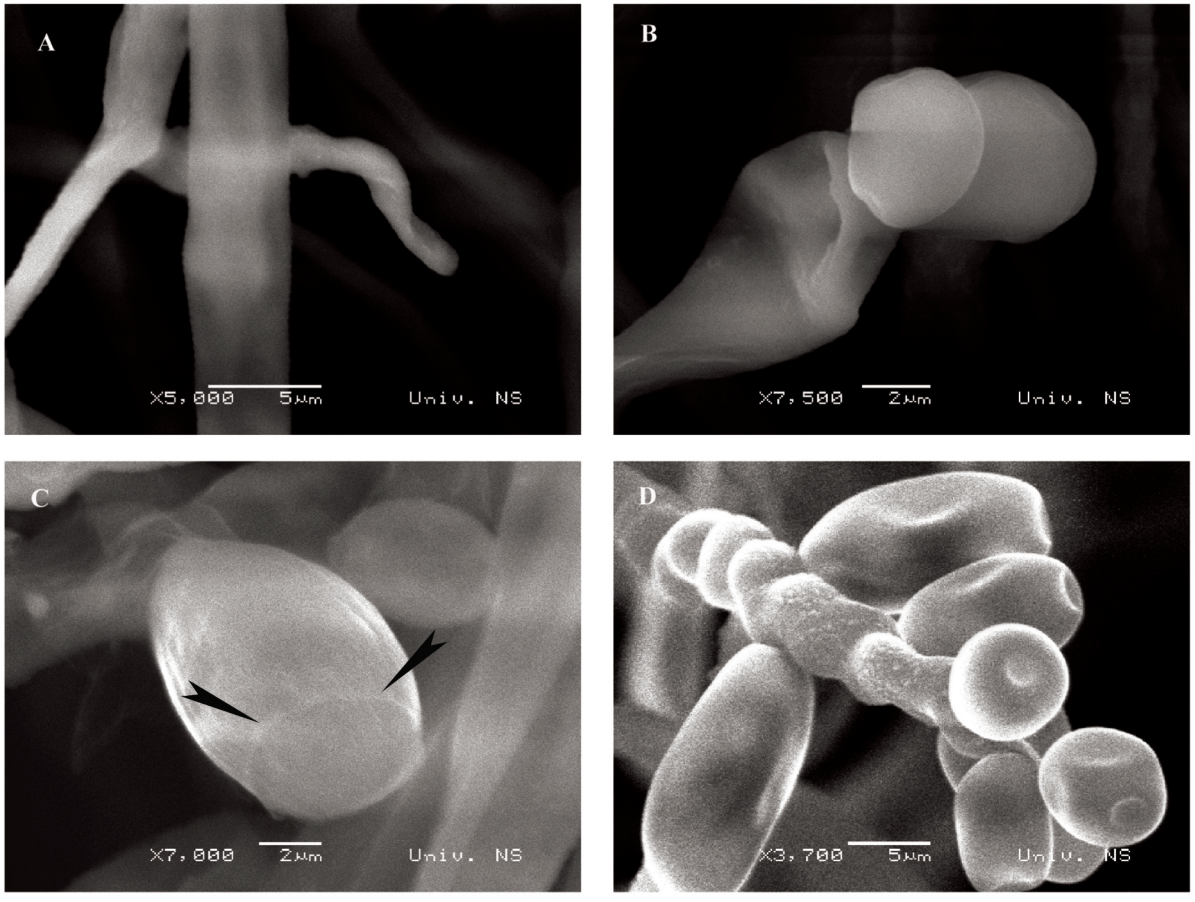

Fig. 4. Morphological changes of *Bipolaris spicifera* conidiogenous apparatus influenced by *Nepeta rtanjensis* essential oil (SEM): A,B. Smooth, sterile or conidiophores with only terminal conidia-0.8 µg ml^{-1} essential oil added. C. Type of conidia without pore and wall rupture (arrow). D. Negative control-typical, roughed, zig-zag conidiophore with poroconidia.

treated samples. The high fungicidal activity of the tested oil was proven with a MIC value (1.0 µg ml^{-1}) and the MIC value for the referent antifungal agent Bifonazole was much higher (10.0 µg ml^{-1}). Espinel-Ingrof et al. (2002) tested the optimal conditions for determining the MICs for uncommon molds, including *B. spicifera,* and reported that the MICs for amphotericin B ranged from 0.06 to 8 µg ml^{-1}, for itraconazole from 0.03 to 8 µg ml^{-1}, for posaconazole from 0.007 to 8 µg ml^{-1}, for ravuconazole from 0.2 to 8 µg ml^{-1} and for voriconazole from 0.12 to 8 µg ml^{-1}. Essential oils are known to cause morphophysiological changes in fungi through a lack of sporulation, depigmentation and aberrant development of conidiophores (eg. Sharma and Tripathi, 2008; Moreira et al., 2010). The most significant documented morphophysiological changes in our investigation included demelanization (bleaching) and an aberrant conidial apparatus of *B. spicifera*. Further investigations are required to determinate whether depigmentation is a result of an inhibition of a melanin biosynthesis or melanin bleaching phenomenon. Reinoculation of hyaline *B. spicifera* on sterile Petri dishes with MEA suggested that depigmentation was reversible. Essential oils are known to interfere with the cell metabolism of fungi. Many components of essential oils can act as regulators of intermediary metabolism; they can change the membrane structure and interfere with the nutrient uptake from the medium; they can affect enzyme

synthesis at nuclear or ribosomal level, or they can substitute the limiting factor in intermediary metabolism (Fries, 1973). Previous investigations of *N. rtanjensis* essential oil's chemical composition showed the presence of phenolic and terpenoid compounds with 4*aα*, 7*α*, 7*aβ*-nepetalactone as the major component (Ljaljević-Grbić et al., 2008). According to Mossier et al. (1993) phenolic derivates are well-known causes of depigmentation. Experiments with other human pathogenic fungi suggested that melanins are not essential for fungal growth but appear to be important as a virulence factor. The mechanisms by which melanins enhance virulence in fungi are yet to be determined, but it has been reported that pigmented cells of *Aspergillus fumigatus* (Tsai et al., 1997), *Exophiala dermatidis* (Dixon et al., 1992) and *Cryptococcus neoformans* (Kwon Chung et al., 1982) are more virulent than hyaline cells. According to Polak (1999), the loss of the production of melanin significantly reduces the pathogenic power of Dematiaceae (24). The present study showed the *in vitro* antifungal activity of *N. rtanjensis* essential oil against *B. spicifera*. *In vitro* data may be helpful in determining the potential usefulness of the plants for treatment of *Bipolaris* infections.

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REFFERENCES

Alcorn, J.L. (1988). The taxonomy of "*Helminthosporium*" species. *Annu. Rev. Phytopathol.* **26**, 37-56.

Babitskaya, V.G., Shcherba, V.V. and *N.V. Lkonnikova* (2000). Melanin complex of the fungus. *Inonotus obliquus*. *Appl. Biochem. Microbiol*. **36**, 377-381.

Bava, A.J., Fayad A., Cespedes, C. and *M. Sandoval* (2003). Fungal peritonitis caused by *Bipolaris spicifera*. *Med. Mycol.* **41**, 529-531.

Butler, J.M. and *A.W. Day* (1998). Fungal melanins: a review. *Can. J. Microbiol.* **44**, 1115–1136.

Butler, J.M., Day, A.W., Henson, J.M. and *N.P. Money* (2001). Pathogenic properties of fungal melanins. *Mycologia* **93**, 1-8.

*Buzina, W., Braun, H., Schimpl, K.*and *H. Stammberger* (2003). *Bipolaris spicifera* causes fungus balls of the sinuses and triggers polypoid chronic rhinosinusitis in an immunocompetent patient. *J. Clin. Microbiol.* **41,** 4885-4887.

Castelnuovo, P., De Bernardi, F., Cavanna, C., Pagella, F., Bossolesi, P., Marone, P. and *C. Farina* (2003). Invasive fungal sinusitis due to *Bipolaris hawaiiensis*. *Mycoses* **47,** 76-81.

Dixon, D.M., Migliozzi, J., Cooper, C.R., Solis, O., Breslin, B. and *P.J. Szaniszlo* (1992). Melanized and non-melanized multicellular form mutants of *Wangiella dermatidis* in mice: mortality and histopathological studies. *Mycoses* **35,** 7-21.

Espinel-Ingroff, A., Chaturvedi, V., Fothergill, A. and *M.G. Rinaldi* (2002). Optimal testing conditions for determining MICs and minimum fungicidal concentrations of new and established antifungal agents for uncommon molds: NCCLS collaborative study. *J. Clin. Microbiol.* **40**, 3776-3781.

Fries, N. (1973). Effects of volatile organic compounds on the growth and development of fungi. *Trans. Br. Mycol. Soc*. **60**, 1-21.

Hemashettar, B.M., Veerappa, T.S., Verma, P.V., Hanchinamani, S., Patil, C.S. and *A. Thammayya (1992)*. Mycotic keratitis caused by *Bipolaris spicifera*. *Indian. J. Pathol. Microbiol.* **35**, 274-227.

Ishii, H. (1995). Monitoring of fungicide resistance in fungi: biological to biochemical approaches. In: *Molecular Methods in Plant Pathology* (Eds. S.U. Singh and P.R. Singh), 483-495. Lewis Publisher Boca Ratton.

Kawamura, C., Moriwaki, J., Kimura, N., Fujita, Y., Fuji, S., Hirano, K., Koizumi, S. and T. Tsuge (1997). The melanin biosynthesis genes of *Alternaria alternata* can restore pathogenicity of the melanin deficient mutant of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* **10**, 445 – 453.

Kwon Chung, K.J., Polacheck, K.J.I. and *T.J Popkin* (1982). Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *J Bacteriol*. **150**, 1414-1421.

Latham, R.H. (2000). *Bipolaris spicifera* meningitis complicating a neurosurgical procedure. *Scand. J. Infect. Dis.* **32**, 102-103.

Ljaljević Grbić, M., Stupar, M., Vukojević, J., Soković, M., Mišić, D., Grubišić, D and *M. Ristić* (2008). Antifungal activity of *Nepeta rtanjensis* essential oil. *J. Serb. Chem. Soc*. **73**, 961–965.

Mc Ginnis, M.R., Campbell, G., Gourley, W.K. and *H.L. Lucia (1992)*. Phaeohyphomycosis caused by *Bipolaris spicifera*: an informative case. *Eur J. Epidemiol.*383-386.

Moore, M.L., Collins, G.R., Hawk, B.J. and *T.S. Russell* (2001). Disseminated *Bipolaris spicifera* in a neonate. *J. Perinatol.* **21,** 399-401.

Moreira, A.C.P., Lima, E.O., Wanderley, P.A., Carmo, E.S. and *E.L. de Souza* (2010). Chemical composition and antifungal activity of *Hyptis suaveolens* (L.) Poit leaves essential oil against *Aspergillus* species. *Braz. J. Microbiol.* **41**, 28-33.

Mossier., D.B., Fitapatrick, T.B., Hori, Y. (1993). Disorder of pigmentation. *Dermatology in general medicine. 4th ed.* (Eds. T.B. Fitapatrick, A.Z. Eisen and K. Wolf) 949. McGraw-Hill, New York.

Nosanchuk, J.D. and *A. Casadevall* (2006). Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. *Antimicrob. Agents. Chemother.* **50**, 3519-3528.

Ogden, P.E., Hurley, D.L. and *Cain, P.T.* (1992). Fatal fungal endarteritis caused by *Bipolaris spicifera* following replacement of the aortic valve. *Clin. Infect. Dis.* **14,** 596-598.

Pandey, D.K., Tripathi N.N., Tripathi, R.D. and *S.N. Dixit* (1982). Fungitoxic and phytotoxic properties of the essential oil of *H. suaveolens*. *Zeit. Pflazenkran. Pflanzensch*. **89**, 344–349.

Polak, A. (1999). The past, present and future of antimycotic combination therapy. *Mycoses* **42**, 355–370.

Romero-Martinez, R., Wheeler, M., Guerrero-Plata, A., Rico, G. and *H. Torres-Guerrero* (2000). Biosynthesis and function of melanin in *Sporothrix schenckii*. *Infect. Immun.* 67, 2845-2853.

Saha, R. and *Das, S.* (2005). *Bipolaris* keratomycosis. *Mycoses* **48**, 453-455.

Sharma, N. and *A. Tripathi,* (2008). Effect of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. *Microbiol. Res.* **163**, 337-344.

Stojanović, G., Radulović, N., Lazarević, J., Miladinović, D. and *D. Đoković,* (2005). Antimicrobial Activity of *Nepeta rtanjensis essential oil. J. Essent. Oil. Res.* **17**, 587- 589.

Straka, B.F., Cooper, P.H. and *B.A. Body* (1989). Cutaneous *Bipolaris spicifera* infection. *Arch Dermatol.* **125,** 1383-1386.

Taguchi, K., Kawabata, T., Wakayama, M., Oharaseki, T., Yokouchi, Y., Takahashi, K., Naoe, S., Ogoshi, T., Iwabuchi, S., Shibuya, K. and *K. Nishimura* (2004). A case of allergic fungal sinusitis caused by *Bipolaris spicifera*. *Nippon. Ishinkin .Gakkai. Zasshi.* **45**, 239-245.

Tsai, H.F., Chang, Y.C., Washburn, G.R. and *K.J. Kwon Chung* (1997). *Aspergillus fumigatus arp1* modulates conidial pigmentation and complement deposition. *Mol Microbial* **26**, 175-183.

Vivek, K.B., Jung, I.Y. and *C.K. Sun* (2009). Antifungal potential of essential oil and various organic extracts of *Nandina domestica* Thunb. against skin infectious fungal pathogens. *Appl. Microbiol. Biotechnol.* **83**, 1127–1133.

Waurzyniak, B.J., Hoover, J.P., Clinkenbeard, K.D. and *R.D. Welsh* (1992). Dual systemic mycosis caused by *Bipolaris spicifera* and *Torulopsis glabrata* in a dog. *Vet. Pathol.* **29,** 566-569.

Youngchim, S., Morris-Jones, R., Hay, R.J. **and** *A.J. Hamilton* **(**2004**).** Production of melanin by *Aspergillus fumigatus*. *J. Med. Microbiol.* **53**,175-181.