



(51) International Patent Classification:

A01N 63/04 (2006.01)

(21) International Application Number:

PCT/HU2019/050002

(22) International Filing Date:

16 January 2019 (16.01.2019)

(25) Filing Language:

Hungarian

(26) Publication Language:

English

(30) Priority Data:

P1800012 16 January 2018 (16.01.2018) HU

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(81) Designated States (unless otherwise indicated, for every

kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every

kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHOD FOR THE TREATMENT OF GRAPEVINE DISEASES

(57) Abstract: The invention relates to a method for the prevention and/or treatment of grapevine trunk diseases, the method comprising treating the plant with a mycoparasitic Trichoderma strain or a composition containing a mycoparasitic Trichoderma strain. The invention also relates to Trichoderma strains suitable to treat grapevine trunk diseases.



METHOD FOR THE TREATMENT OF GRAPEVINE DISEASES

FIELD OF THE INVENTION

The present invention relates to a method for the prevention and / or treatment of Grapevine Trunk Diseases comprising treating the plant with a mycoparasitic *Trichoderma* strain or a composition containing it. The invention also relates to *Trichoderma* strains which are suitable for the treatment of Grapevine Trunk Diseases.

BACKGROUND OF THE INVENTION

The Grapevine Trunk Diseases (GTDs) lead to early death of the grapevine. It is a complex disease, as several plant pathogenic fungi can be the causative agent, and the development of symptoms and the severity of the disease are greatly influenced by other environmental factors. GTDs are caused by many different Ascomycetes fungi, including *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* and other *Phaeoacremonium* sp., *Eutypa lata*, *Diplodia seriata*, *Phomopsis viticola*, as well as Basidiomycetous fungi such as *Fomitiporia mediterranea*. Dying can be caused by a combination of several fungal species. The pathogen species and the distribution of pathogens depend on the plant species, varieties, rootstock, climate, etc. Esca disease complex, Petri disease, *Eutypa*, *Botryosphaeria*, *Phomopsis* dieback, black dead arm (BDA) and the black foot disease are considered the major GTDs worldwide (Kovács 2017).

The effective control of GTD faces several problems, as no grapevine cultivar known to be resistant to GTD, and effective chemical pesticide is not available. The protection is restricted currently mainly to the prevention (healthy propagating material, prevention of infection through pruning wounds).

Trichoderma species have rapid growth, and they produce aerial mycelia. Their optimum growth temperature is usually 30°C. Conidiophores are formed on the hyphae; conidia are single-celled with white or green colour (Kövcics, 2009), and they are located in scattered or concentric circles on the medium. Conidia can have various form and size. The thin hyphae are indicated in the genus name from Greek „tricho” (hair) word.

Trichoderma species are potential biopesticides, and some species are used as biopesticides against plant pathogen fungi (Weindling, 1932). The species generally have rapid growth, so they displace the plants pathogens from the plant environment (spatial parasitism). As a mycoparasite, they are able to destroy the hyphae of other fungi (e.g. plant pathogens). In addition to their spatial parasitic and mycoparasitic activity, they are also able to effectively inhibit the growth of other microbes by selecting mycotoxic compounds (antibiosis) (Kubicek et al., 2007).

The results of the use of *Trichoderma* species were summarized in the WINETWORK project (www.winetwork.eu) (<http://www.winetwork-data.eu/intranet/libretti/0/libretto16735-01-1.pdf>, published August 30, 2017, cited as of 11 December 2017), and it was stated that the *Trichoderma* species and strains tested as biocontrol agent against GTD were *Trichoderma atroviride*, *Trichoderma asperellum*, *Trichoderma gamsii* and *Trichoderma harzianum* species. It was highlighted, that *Trichoderma* species and strains possess several (species or even strain-specific) mechanism of biocontrol activity which are regulated at gene levels. *Trichoderma* species are used in prevention, they have no curative effect against GTDs, but are effective, for example, in preventing infection of pruning wounds. It is clear from the summary that *Trichoderma* containing products

should be used on pruning wounds or on propagating materials (grafts) during the critical period after pruning (2-8 weeks), more precisely between dormancy period and start of the bleeding, to prevent infection with pathogens. Treatment of pruning wounds are recommended to be started after planting and then annual treatment is required. It was also stated that the efficacy of *Trichoderma* species was not fully supported by field experiments, and that controlled experiments were needed.

The inefficiency may be due to the applied products containing *Trichoderma* species not originated from grapevines, or that the *Trichoderma* strains used could colonize only near to the surface of the wounds if they are capable of colonization at all. According to the summary, the effectiveness of *Trichoderma* species was only partial against the GTDs.

Di Marco et al. similarly reported, that eradication of the esca pathogen currently was not possible. They used *T. harzianum* T39 strain on the pruning wounds, which prevented black dead arm and reduced *Phaeomoniella chlamydospora* infection. The authors emphasized that *Trichoderma* treatment provided effective prevention in the case of uninfected grapes. Di Marco et al. also investigated whether *Trichoderma* could be used as a plant growth promoter prior to callus formation, but their results had not supported this application (Di Marco et al. Experiments on the Control of Esca by *Trichoderma*. 43, 108-115).

Chaverri et al. highlighted the need for accurate, genetic identification of *Trichoderma* species and strains if they are to be used in plant protection products. It was found in their investigations, that within the *Trichoderma* genus, the morphology of the species can be the same, but their biological characteristics are very different. The members of the *harzianum* species complex also show great morphological similarity, but their geographical distribution, their ecological roles, and lifestyles (e.g. living in the soil or as endophytes) are different. It has been found, for example, that the most commonly tested T22 *T. harzianum* strain (also used in plant protection products and plant growth promoters) is *Trichoderma afroharzianum*. Detailed genetic analysis has also shown that the CGMCC 1780 *T. harzianum* strain, described as an antifungal agent, is actually a mixture of two different species, one of which produces trichodermin mycotoxin, therefore its use as a pesticide is not possible.

The present inventors have also proven earlier that some *Trichoderma* strains can colonize pruning wounds, thus providing protection through the pruning surface against fungi that infect the plant (e.g., Cs. Kovács. PhD Thesis, University of Debrecen Kálmán Kerpely Doctoral School of Plant Production and Horticulture, 2017 and Kovács, C., Peles, F., Sándor, E.: Potential Biopesticide against GTD pathogens isolated from asymptomatic grapevines. Plant Protection Sciences, Berlin, 2015).

There is a need for compositions containing a *Trichoderma* strain that is genetically identified, harmless to humans, easy to use on vine plants, and can effectively prevent and / or treat grape trunk diseases.

BRIEF DESCRIPTION OF THE INVENTION

To our best knowledge, prior to the invention no method existed, wherein grapevine plants (*Vitis vinifera* L.) were lastingly and extensively colonized with a *Trichoderma* strain by contacting the grapevine plant with the *Trichoderma* strain through the root or any other intact tissue of the grapevine plant. Due to the ability of the *Trichoderma* strains according to the invention of colonizing grapevine (trunk, cordon arm, branches, roots) in a lasting and extensive manner, the number of the necessary *Trichoderma* treatments may be decreased. Treatment

of the grapevine plant is made considerably easier by the ability of the *Trichoderma* strains according to the invention of providing effective and long lasting protection not only through the pruned surfaces, so that in contrast to other *Trichoderma* strains known to be used for preventive treatment, *Trichoderma* strains according to the invention may be applied to roots and other intact, undamaged parts of the plant, because the *Trichoderma* strains according to the invention are capable of extensive and long lasting colonization when applied this way.

The inventors have demonstrated that some of the *Trichoderma* strains isolated by them are capable of growing in a wide range of temperatures, are capable of total inhibition (killing) pathogens causing grapevine trunk diseases in vitro. It has been demonstrated in field experiments that the *Trichoderma* strains according to the invention are useful in the prevention and treatment of trunk diseases, even when used before planting or on propagation material. The strains according to the invention colonized the tested plants extensively, even in places at a considerable distance from the place of application and in a long lasting manner. The strains according to the invention facilitated plant growth and had a positive effect on the quality of the fruit as well.

Method for the treatment of grapevine trunk disease (GTD), comprising contacting the grapevine plant with a mycoparasitic *Trichoderma* strain, wherein

- the grapevine is contacted through an intact tissue thereof with a strain selected from strains belonging to the group of species comprising: *Trichoderma harzianum* species group, *Trichoderma viride*,
- thereby woody tissue of the grapevine is colonized with the *Trichoderma* strain such that the *Trichoderma* strain may still be detected in the woody tissue at least five months, preferably at least eight months, most preferably at least 12 months after said contacting,
- thereby a pathogenic fungus causing GTD is controlled in the woody tissue of the grapevine by the at least one *Trichoderma* strain,

wherein in the grapevine contacted with the at least one *Trichoderma* strain, at least one symptom of GTD is decreased or eliminated within three months, preferably two months, most preferably one month following said contacting,

wherein the *Trichoderma* strain forms appressorium against *Diplodia seriata* and/or *Neofusicoccum parvum*, and its Biocontrol-Index (BCI) value is at least 90%, preferably at least 95%, most preferably 100%,

and wherein the *Trichoderma* strain may be provided by a method comprising the steps of

- (a) providing a tissue sample comprising a mycoparasitic *Trichoderma* strain, from an internal, woody tissue of a grapevine comprising GTD causing pathogenic fungi, preferably *Diplodia seriata* and/or *Neofusicoccum parvum*, wherein the grapevine does not show leaf symptoms of GTD,
- (b) isolating the *Trichoderma* strain from the sample of (a),
- (c) preparing a culture from the isolate of (b).

Preferably, the grapevine is contacted with the *Trichoderma* strain through its root,

- to colonize woody tissue of the grapevine plant other than the root with the *Trichoderma* strain such, that the *Trichoderma* strain is detectable in the woody tissue other than the root at least five months after, preferably at least eight months after, most preferably at least 12 months after said contacting,
- to control pathogenic fungi causing GTD in woody tissue of the grapevine plant, preferably in the root and in woody tissue other than the root.

Preferably the grapevine plant is a graft with roots, which is contacted with the *Trichoderma* strain by soaking through the root before planting. When an already planted grapevine plant is to be contacted with the *Trichoderma* strain, said contacting is carried out by applying a composition comprising the *Trichoderma* strain on the soil around the plant or into the soil (e.g. delivering it into the soil by using high pressure).

5 Preferably the *Trichoderma* strain is detectable in a woody tissue at a distance of at least 15 cm, preferably at least 20 cm from the upper end of the root (or the root collar) five months after said contacting, most preferably detectable from a woody tissue of the plant one year after, more preferably two years after, even more preferably more than two years following said contacting.

10 The method preferably comprises contacting the grapevine plant with at least two *Trichoderma* strains, wherein the two *Trichoderma* strains are selected from DE_TR04, DE_TR05 and derivatives thereof.

Preferably the trunk disease is black arm death.

Most preferably the trunk disease is a disease associated with *Diplodia seriata* and/or *Neofusicoccum parvum* infection.

15 Most preferably, the grapevine plant is contacted with the *Trichoderma* strain once in every two years, preferably every three years, most preferably once in the lifetime of the plant.

Preferably the symptom of GTD which has decreased or has been eliminated three months, two months or one month after said contacting, does not increase or appear again within one year, preferably within two years following said contacting.

Trichoderma strain, which

- 20 - is selected from the strains belonging to the species of the group comprising: *Trichoderma harzianum* species group, *Trichoderma viride*,
- upon contacting it with the root of the grapevine, is capable of colonizing woody tissue other than the root of the grapevine such that the *Trichoderma* strain may be detected in the woody tissue other than the root even at least five months, preferably at least eight months, most preferably at least 12 months after said contacting,
- 25 - forms appressorium against *Diplodia seriata* and/or *Neofusicoccum parvum*, and has a Biocontrol-Index (BCI) value of at least 90%, preferably at least 95%, most preferably 100%,

and wherein the *Trichoderma* strain may be provided by a method comprising the steps of

- (a) providing a tissue sample from an inner woody tissue of a grapevine showing no leaf symptom of GTD and comprising a pathogenic GTD fungus, preferably *Diplodia seriata* and/or *Neofusicoccum parvum*, wherein
- 30 the sample comprises a mycoparasitic *Trichoderma* strain,
- (b) isolating the *Trichoderma* strain from the sample of (a),
- (c) preparing a culture from the isolate of (b).

35 Preferably, the *Trichoderma* strain is detectable in a woody tissue at a distance of at least 15 cm, preferably at least 20 cm from the top of the root after three to five months, preferably two to five months, most preferably five months following said contacting, most preferably the *Trichoderma* strain is detectable in all woody tissues of the plant, most preferably the *Trichoderma* strain is detectable in any woody tissue of the plant even one year after, preferably two years after, more preferably more than two years after said contacting.

Most preferably the at least one *Trichoderma* strain is selected from: DE_TR04 deposited with the Natio-

nal Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, DE_TR08 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001458, and derivatives thereof.

5 More preferably, the at least one *Trichoderma* strain is selected from: DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, and derivatives thereof.

Use of a *Trichoderma* strain according to the invention for facilitating plant growth, comprising contacting the plant with the *Trichoderma* strain. Preferably the plant is contacted with the *Trichoderma* strain
10 once in a year, in every two years or once during the lifetime of the plant.

Grapevine propagation material, wherein the grapevine propagation material is (has been) contacted with a *Trichoderma* strain according to the invention, preferably wherein the propagation material is a graft having roots, wherein said contacting is (has been) carried out on the root. Due to contacting, the *Trichoderma* strain colonizes woody tissue of the grapevine plant developing from the propagation material in such a way that the
15 *Trichoderma* strain is detectable in the woody tissue even at least five months, preferably at least eight months, most preferably at least 12 months after said contacting, thereby rendering the grapevine resistant to pathogenic GTD fungi.

A composition, comprising a *Trichoderma* strain according to the invention and an agriculturally acceptable carrier or excipient, preferably comprising a *Trichoderma* strain selected from the group consisting of
20 DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, DE_TR08 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001458, and derivatives thereof, most preferably comprising at least two *Trichoderma* strains selected from DE_TR04 deposited with the National Collection of Agricultural
25 and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, and derivatives thereof.

Preferably in step (a) of the method for providing the *Trichoderma* strain, the sample from the grapevine comprises a pathogenic fungus causing trunk disease, preferably *Diplodia seriata* and/or *Neofusicoccum parvum*.

Preferably in step (c) of the method for providing the *Trichoderma* strain, the culture does not comprise
30 microbes apart from microbes belonging to the *Trichoderma* genus, most preferably does not comprise any other *Trichoderma* strain apart from the *Trichoderma* strain isolated in step (b) (i.e. a pure *Trichoderma* stock culture).

In a preferred embodiment the composition comprises the strain DE_TR04 or a derivative thereof, the strain DE_TR05 or a derivative thereof and the strain DE_TR08 or a derivative thereof.

The invention provides a *Trichoderma* strain selected from the group consisting of the strain DE_TR04
35 with the accession No. NCAIM (P) F 001456, and any derivative thereof, the strain DE_TR05 with the accession No. NCAIM (P) F 001457, and any derivative thereof, the strain DE_TR08 with the accession No. (P) F 001458, any derivative thereof, deposited with the National Collection of Agricultural and Industrial Microorganisms (Szent István University, Faculty of Food Science, H-1118 BUDAPEST, Somlói út 14-16, Hungary) on 12 Dec-

ember 2017 The *Trichoderma* strain in the method and/or composition according to the invention is preferably selected from the group consisting of DE_TR04 deposited on 12 December 2017 with the National Collection of Agricultural and Industrial Microorganisms (Szent István University, Faculty of Food Science, H-1118 BUDAPEST, Somlói út 14-16, Hungary) under the accession No. NCAIM (P) F 001456, and any derivative thereof, DE_TR05 with the accession No. NCAIM (P) F 001457, and any derivative thereof, DE_TR08 with the accession No. (P) F 001458, and any derivative thereof. More preferably the *Trichoderma* strain is a *Trichoderma* strain selected from DE_TR04 and derivative thereof, DE_TR05 and derivative thereof. Most preferably the *Trichoderma* strain is selected from DE_TR04 and DE_TR05.

The strains according to the invention may be applied to the pruning wounds of the grapevine plant. The root is preferably an intact root without damage (not cut back).

The method and strains according to the invention are also useful for preventive treatment.

SHORT DESCRIPTION OF THE FIGURES

Figure 1: Macromorphology of *Trichoderma harzianum* strains (DE_TR04 left, DE_TR05 right) on potato-dextrose agar medium.

Figure 2: Macromorphology of *Trichoderma viride* UD_TR08 strain on potato-dextrose agar medium.

Figure 3: Phylogenetic tree of *Trichoderma* isolates based on ITS sequences.

The length of branches is proportional to the number of nucleotide differences in the sequences, the scale is under the dendogram. The blue number shows the results of the bootstrap analysis (it means we had 1000 replicates) next to the branches. We indicated more than 50% values on the tree. The Accession Number is before species name. TR01-TR10 show our isolates.

Figure 4: Tef1 phylogenetic tree of *Trichoderma* isolates

The length of branches is proportional to the number of nucleotide differences in the sequences, the scale is under the dendogram. The blue number shows the results of the bootstrap analysis (it means we had 1000 replicates) next to the branches. We indicated more than 50% values on the tree. The Accession Number is before species name. TR01-TR10 show our isolates.

Figure 5: Phylogenetic trees of *Trichoderma harzianum* strains based on tef1 (A) and chi18-5 (B) genes

The length of branches is proportional to the number of nucleotide differences in the sequences, the scale is under the dendogram. The blue number shows the results of the bootstrap analysis (it means we had 1000 replicates) next to the branches. We indicated more than 50% values on the tree. The Accession Number is before species name. TR01-TR10 show our isolates.

Figure 6: Mycelial growth of TR01-TR05 *T. harzianum* strains between 18,5-37°C.

Figure 7: Mycelial growth activity of TR07-TR10 *Trichoderma* strains

Figure 8: Mycelial growth rate of *Trichoderma harzianum* (TR01, DE_TR05, TR09), *Trichoderma viride* (DE_TR08) and *Trichoderma orientale* (TR06).

Figure 9: Mycelial growth of *Trichoderma* isolates at 5°C.

Figure 10: Mycelial growth activity of *Trichoderma harzianum* (TR04, DE_TR05) and *Trichoderma orientale* (TR06) at 12°C.

Figure 11: Mycoparasitic ability of DE_TR04, DE_TR05 *T. harzianum* and TR06 *T. orientale* strains

against *D. seriata* pathogen.

Figure 12: Mycoparasitic ability of DE_TR04, DE_TR05 *T. harzianum* and TR06 *T. orientale* strains against *N. parvum* pathogen.

Figure 13: Tef1 Phylogenetic trees of *Trichoderma* (TRI01-04) isolates from Tokaj Wine Region

The length of branches is proportional to the number of nucleotide differences in the sequences, the scale is under the dendrogram. The blue numbers shows the results of the bootstrap analysis (it means we had 1000 replicates) next to the branches. We indicated more than 50% values on the tree. The Accession Number is before species name. DE_TR05, TR06 show our used isolates, TRI01, TRI03, TRI04 are the re-isolated *Trichoderma* species.

Figure 14: Tef1 Sequence alignment detail of the used, extype strains and re-isolated *Trichoderma* isolates from Tokaj Wine Region.

Figure 15: Tef1 Sequence alignment detail of the used, extype strains and re-isolated *Trichoderma* isolates from Villány Wine Region.

Figure 16: Phylogenetic trees of used and deponated *Trichoderma* isolates based on tef1 gene sequence

The length of branches is proportional to the number of nucleotide differences in the sequences, the scale is under the dendrogram. The blue number shows the results of the bootstrap analysis (it means we had 1000 replicates) next to the branches. We indicated more than 50% values on the tree. The Accession Number is before species name, DE_TR05, TR06 show our, used isolates Sz 01-03, SZ 05-08 are the re-isolated *Trichoderma* species.

Figure 17: Szálka plantation. After the plantation, 4 soaked in *Trichoderma*-solution trunks were collected in autumn for *Trichoderma* re-isolation (A). Isolation place of the *Trichoderma* strains. T4 shows the 4th trunk sample, the /1 - /5 indicate the isolation place (B). Sampling was from inside of the rootstock and the noble ca. 5 centimetres.

Figure 18: *Trichoderma* strains from different trunks and sampling place.

Figure 19: Percentage of the destroyed trunks in case of the different treatments in average of the planted varieties (Villány Wine region, 2015 plantation).

Figure 20: Effect of the different treatments for the plant growth intensity following 12-24 hours after one year of the plantation. The results show the average of the different, planted varieties (Villány Wine region, 2015 plantation).

Figure 21: Tef1 Phylogenetic tree of *Trichoderma harzianum* subclade strains (TR01-10), reisolated strains (TRI01-04) and the deponated strains with Maximum Likelihood analysis. The length of branches is proportional to the number of nucleotide differences in the sequences, the scale is under the dendrogram. The blue number shows the results of the bootstrap analysis (it means we had 1000 replicates) next to the branches. We indicated more than 50% values on the tree. The Accession Number is before species name.

Figure 22: Sequence alignment of the *Trichoderma harzianum* subclade strains (TR01-10), some, re-isolated strains (TRI01-04) and deponated strains.

Figure 23: Deponated tef1 sequence alignment of UD_TR04 *Trichoderma harzianum* subclade strain (and Tr01-02, 07, 10), *Trichoderma harzianum* and *Trichoderma simmonsii* species. The numbers above the columns

show the number of the sequences, the big letters below the columns indicate the consensus sequences.

Figure 24: Separation of *Trichoderma* strains based on microsatellite PCR with TaSSR1 and ThSSR1 primers. TaSSR1 (left) and ThSSR1 (right) PCR at 55°C annealing temperature. Samples: 1. and 4. DE_TR04; 2 and 5 DE_TR05; 3rd and 6th DE_TR08.

5 Figure 25: Separation of *Trichoderma* strains based on microsatellite PCR with ThSSR6, TvSSR1 and TvSSR5 primers. ThSSR6 (left), TvSSR1 (middle) and TvSSR5 (right) 55°C annealing temperature. Samples: 1., 4., 7. DE_TR04; 2., 5., 8. DE_TR05; 3., 6., 9. DE_TR08.

Figure 26: Separation of *Trichoderma* strains based on microsatellite PCR with ThSSR4 primers. ThSSR4 57 °C annealing temperature. Samples: 1. DE_TR05; 2. DE_TR08.

10 Figure 27: Separation of *Trichoderma* strains based on microsatellite PCR with ThSSR4 primers. ThSSR4 57 °C annealing temperature. Samples: 1. DE_TR04; 2. DE_TR05; 3. DE_TR08.

DETAILED DESCRIPTION OF THE INVENTION

15 The method of the invention is suitable for the prevention and / or treatment, preferably treatment, of grapevine trunk diseases.

The *Trichoderma* strains used in the method were isolated from internal woody tissues of the cordon of grapevine plants, Furmint variety, in a Hungarian terroir, without detectable GTD leaf symptoms and The strains were deposited according to the Budapest Treaty with the National Collection of Agricultural and Industrial Microorganisms (Szent István University, Faculty of Food Science, H-1118 BUDAPEST, Somlói út 14-16, Hungary) on 12. December 2017. The deposit number of DE_TR04 strain NCAIM (P) F 001456, the deposit number of DE_TR05 strain NCAIM (P) F 001457, and the deposit number of DE_TR08 strain NCAIM (P) F 001458. *In vitro* experiments proved that the strains had very high (100%) Biocontrol Index (BCI) against known GTD pathogens (*Diplodia seriata*, *Neofusicoccum parvum*), i.e., destroyed the GTD pathogens and appressorium formation was observed on the microscope images. BCI is a good indicator in laboratory conditions, because it is showing, how much area the biocontrol (BC) strain i.e. the mycoparasite (in our case *Trichoderma*) occupies in the Petri-dish in a confrontation experiment. BCI is the rate of the size of the area occupied by BC and the area with the parasite fungi (calculated in %) (Szekeres et al. 2006). The strains are therefore characterized by hyperparasitism.

30 The *Trichoderma* strains according to the invention can grow at a wide temperature range on potato dextrose agar (PDA) medium. Their temperature optimum is around 30°C, but they can grow at 5°C, 12°C and 37°C. Their morphology is characterized by slightly elongated spherical conidia and conidia forming in scattered or concentric circles. The strains can be stored on sloped agar at 4°C and -80°C in 20% glycerol solution and lyophilized. It was shown that our strain mix grew faster and sporulated earlier under the same conditions, than *Trichoderma atroviride* (strain I-1237), which found in the biofungicide, ESQUIVE.

35 The isolation and culture of *Trichoderma* strains has been described in the literature and can be accomplished, for example, as described in the Examples section. The *Trichoderma* strains according to the invention were isolated from the internal woody part of the non GTD symptomatic trunk, with detected GTD pathogens inside, preferably *Diplodia seriata* and / or *Neofusicoccum parvum* fungi and then cultured.

The Biocontrol Index value of *Trichoderma* strains can be determined by the method described in the

literature and in the Examples section, by in vitro experiments.

DE_TR04 strain produces diffusing yellow pigment on potato dextrose agar medium.

As used herein, a derivative of the DE_TR04 strain refers to fungi of *Trichoderma harzianum* species, including subclass (lixii) subclade, derived from the strain DE_TR04 (generated by the cultivation of DE_TR04 strain), as a progeny, forms appressorium against *D. seriata* and *Neofusicoccum parvum* and has a Biocontrol Index (BCI) at least 90%, preferably 95%, particularly preferably 100%,

when contacted with the root of a grapevine plant, it can colonize the woody tissue beyond the root of a vine plant, in such a way, that the *Trichoderma* strain can be detected in woody tissue other than roots at least five months, preferably at least eight months, particularly preferably at least 12 months, after said contacting.

Preferably, it can be detected in woody tissue at least 15 cm from the top end of the root five months after contact, preferably at least 20 cm apart. Preferably, it can grow on the potato dextrose agar medium at 5°C with an average mycelial growth of at least 1 mm / day, preferably at least 1.5 mm / day, particularly preferably at least 2 mm / day. Preferably, it forms appressorium toward *Fusarium oxysporum* and its BCI is at least 50%, preferably at least 75%, particularly preferably 100% against *F. oxysporum*. Particularly preferably, the *tefl* gene comprises a sequence that is at least 95%, preferably at least 98%, more preferably at least 99%, particularly preferably 100%, identical with SEQ ID NO: 1, wherein there is T at the position corresponding to position 138 of SEQ ID No:1 and there is A at the position corresponding to position 156 of SEQ ID No:1; and / or the *chi18-5* gene comprises a sequence that is at least 95%, preferably at least 98%, more preferably at least 99%, particularly preferably 100% identical with the sequence of SEQ ID NO: 2. Preferably, it comprises six of the following microsatellites, preferably comprising the following microsatellites: TaSSR1 (<100 bp A, B, C), ThSSR1 (211 bp), ThSSR6 (400 bp), TvSSR1 (500 bp), TvSSR5 (198 bp), ThSSR4 (159 bp). Preferably, after contact with the vine, preferably with the intact tissue of the vine, particularly preferably with the intact roots of the vine, at least one symptom of the GTD in the vine is reduced or eliminated three months after treatment, preferably two months, particularly preferably one month.

As used herein, a derivative of the DE_TR05 strain refers to fungi of the *Trichoderma harzianum* species group, including subclade II, which are derived from the strain DE_TR05 (prepared by culturing DE_TR05),

it forms appressorium toward *Diplodia seriata* and *Neofusicoccum parvum* and its Biocontrol Index (BCI) value is at least 90%, preferably 95%, particularly preferably 100% against the two pathogens. In particular, it is capable of colonizing a woody tissue other than the root of a vine by contact with the root of a vine plant, such that the *Trichoderma* strain can be detected from at least five months, preferably at least eight months, particularly preferably at least 12 months, of woody tissue other than roots. Preferably, five months after contacting, it can be detected in a woody tissue at a distance of at least 15 cm from the top end of the root, preferably at least 20 cm apart.

Preferably, after contact with the vine, preferably with the intact tissue of the vine, particularly preferably with the intact roots of the vine, at least one symptom of the capital disease in the vine is reduced or eliminated within three months after treatment, preferably two months, particularly preferably one month.

Preferably, it can grow on potato dextrose agar medium at 5°C with an average mycelium growth of at least 0.5 mm / day, preferably at least 1 mm / day, particularly preferably at least 1.5 mm / day,

and optionally forms appressorium toward *Fusarium oxysporum* and its BCI value is least 50%, preferably at least 75%, particularly preferably 100%.

Preferably, the *tefl* gene comprises a sequence that is at least 95%, preferably at least 98%, more preferably at least 99%, particularly preferably 100% identical with the sequence according to SEQ ID NO: 3 and / or the *chi18-5* gene comprises a sequence that is at least 95%, preferably at least 98%, more preferably at least 99%, particularly preferably 100% identical sequence with the sequence according to SEQ ID NO: 4.

Preferably, it comprises eight or nine of the following microsatellites, preferably comprises the following microsatellites: TaSSR1 (<100bp A, C; 258 bp; 338 bp; 407 bp), ThSSR1 (211 bp), ThSSR6 (390 bp), TvSSR1 (500 bp), TvSSR5 (198 bp), ThSSR4 (159 bp).

As used herein, a derivative of DE_TR08 is a fungus belonging to the species *Trichoderma viride*, which is derived from the strain DE_TR08 (generated by culturing DE_TR08),

It forms appressorium toward *Diplodia seriata* and *Neofusicoccum parvum* and its Biocontrol Index (BCI) value is at least 90%, preferably 95%, particularly preferably 100%.

Preferably, it can grow on the potato dextrose agar medium at 5°C with an average mycelium increase of at least 0.5 mm / day, preferably at least 1 mm / day.

Optionally, it forms appressorium toward *Fusarium oxysporum* and has a BCI value of at least 50%, preferably at least 75%, particularly preferably 100%.

and / or optionally comprises one or more of the following microsatellites, preferably comprises the following microsatellites: TaSSR1 (150 bp), ThSSR1 (213 bp), ThSSR6 (378 bp), TvSSR1 (500 bp), ThSSR4 (<100bp) A, B; 159 bp).

Particularly preferably, when contacted with the root of a vine plant, it is capable of colonizing a woody texture other than the root of the vine plant, such that the *Trichoderma* strain can be detected from woody tissue other than roots at least five months after contact, preferably at least eight months, particularly preferably at least 12 months. Preferably, it can be detected in woody tissue at least 15 cm from the top end of the root five months after contact, preferably at least 20 cm apart.

Preferably, after contacting with the vine, preferably with the intact tissue of the vine, particularly preferably with the intact roots of the vine, at least one symptom of the capital disease in the vine is reduced or eliminated three months after treatment, preferably two months, particularly preferably one month.

The *Trichoderma* strains according to the invention colonize the treated plant very quickly: 5 months after treatment, we were able to isolate the strains from the root level to the top of the sprout after treatment. Three days after the surface treatment of the propagation material, the strain used for treatment was detected from the internal woody tissue. As used herein, the terms "vine", "grapevine" or "grapevine plant" refers to propagating material, grafts, shoots, rootstock, and cittings, particularly the grafts.

The strains were isolated from the woody tissue of vine cordon arm. During the sampling 10-15 cm section was cut so that the vitality and productivity of the trunk did not change significantly. According to one aspect of the present invention, there is provided a method of providing a mycoparasitic *Trichoderma* strain, capable of treating and / or preventing GTD, the method comprising isolating the *Trichoderma* strain from an internal woody tissue of a grapevine plant comprising a GTD pathogen, preferably *Diplodia seriata* and/or

Neofusicoccum parvum, said grapevine not exhibiting foliar symptoms of GTD, preferably not exhibiting any GTD symptom, and culturing the Trichoderma strain, optionally and preferably preparing a culture of the Trichoderma strain not comprising any microbe belonging to a species other than Trichoderma spp., most preferably not comprising any microbe belonging to a strain other than the Trichoderma strain. The term "woody tissue" as used herein, refers to a woody tissue other than a bark, preferably a woody fabric other than a root.

Our strains were able to colonize far from the treatment site the:

- i. cordon arm of older plants (Tokaj test)
- ii. grafts (both rootstock and scion (Legyesbénye test))
- iii. roots (Villány test, Szekszárd test)

iv. the entire woody plant tissue (subject and noble comma, trunk, cordon arm) (Szekszárd vine plantation).

To our best knowledge, experiment iv. was the first test proving that a GTD curative Trichoderma strain, which is suitable for use in a plant protection, was able to colonize the woody parts of the whole plant, following root treatment, even in case of contact with intact plant tissue. It was proved in field experiments, that the strains were capable of colonizing the vine permanently (beyond Furmint varieties, also the Tarcál 26, Hárslevelű, Cabernet Franc, and Kékfrankos), the strains could be re-isolated from the treated plants at least one but up to two years after treatment (the treated plants did not contain the Trichoderma strains before treatment). The long-term colonization is still studied therefore a longer colonization of fungal strains is expected within the grapevine plant.

Reduction of the typical symptoms of GTD on the vegetative organs of the treated plants (which were showing GTD symptoms) was found due to the treatment with the strains of the invention, i.e. the treatment had a curative effect.

The curative effect was also confirmed by isolating our strains from the necrotic woody tissues of the treated grape plants belonging to different varieties. The Trichoderma strains according to the invention are able to control the pathogenic fungi not only on the surface of the pruning wounds but also on the inside of the woody tissue. Trichoderma could be re-isolated following a very long time after one time application (two years later), therefore the use of the solution provided by the present invention can provide a long-term treatment of the disease symptoms, and the destruction and control of pathogenic species. The present invention therefore provides for less treatment, and the annual application of Trichoderma is no longer necessary.

The term "treatment" in the specification, unless the context otherwise requires, is used in a curative meaning, i.e. treating a disease means curative treatment of the disease. Curative treatment may include a reduction in the typical symptoms of the disease, a reduction in the amount of the infectious agent (e.g., a phytopathogenic fungus) in the plant, or a reduction or stopping of the spread of an infectious agent (e.g., a phytopathogenic fungus) in a plant.

Contacting the Trichoderma strains with the vine plant may be carried out at any time of the year, preferably at the end of winter, early spring and / or before planting.

Treatment can be monitored both by examining the colonization of the Trichoderma strain used, detecting the presence of the Trichoderma strain after exposure, and / or the number of symptoms, the severity of symptoms, or the disappearance of the symptoms and / or the decrease of the presence or absence of pathogenic

fungi in the vine.

The reduction of symptoms after contacting with the strains or compositions of the present invention is such, that preferably the viability and / or fertility of the plant do not differ substantially from that of an asymptomatic plant, the symptoms do not substantially reduce the life and / or fertility of the plant. The presence of the pathogenic fungus (the amount of pathogenic fungus present in the plant) after contacting the vine plant with the strains or compositions of the present invention is preferably such that the pathogenic fungus present in the plant does not cause symptoms that significantly reduce the life and / or productivity of the plant. Particularly preferably, the reduction of symptoms and / or the presence of the pathogenic fungus are sustained for at least three months after the application of the present invention, preferably for at least 6 months, preferably at least one year, particularly preferably at least two years or at least three years.

Typical Symptoms of GTDs:

Petri Disease: Typical symptoms are detectable on shoots younger than 1 year. Its main external symptom is stunt plant, chlorotic leaves with necrotic borders. The characteristic change inside the trunk is the dark colouration (brown red/brown necrosis), the black spots or the ring in the woody tissues and the dark-coloured gummy exudates.

Esca Disease Group: Typical in established vineyards. It has two forms: (i) Acute form: "apoplexia", with sudden wilting, falling leaves and wrinkling of the grapes, appearing in mid-summer. The leaves turn pale green to gray-green and dry. This process takes place in a few days. (ii) Chronic form: "black measles", a long-lasting, multi-year process that mainly attacks 8-10 years or older plantations. The most common internal symptom is white rot, where the woody tissue becomes spongy and soft. Necrosis occurs mainly in the case of larger pruning wounds. Leaves have necrotic and chlorotic lesions called "tiger stripes".

The typical symptom of the Eutypa cancer and dieback is the wedge-shaped zoned, brownish, longitudinal necrotic necrosis in the cross-section of cordons and trunks, and the necrotic spots on the leaves.

Dieback caused by Botryosphaeria species can be very diverse and do not appear on all trunks. At first, the leaves may have necrosis, in chlorotic spots becoming dry. Weakened or terminated vegetative development. Cross or longitudinal dark brown coloration can be seen in the xylem tissues. In the case of a heavily infected plant, necrosis can be observed already at the time of bud burst. The bark becomes white, with dark spots. Pycnidia of the Botryosphaeria species develop from dead/cankered wood. The branches start to rot.

Black dead arm: this is a special form within the Botryosphaeria dieback disease group. Typical woody tissue symptoms are brown stripes.

Symptoms of black foot disease appear at the beginning of the vegetation period. The new shoots show weak growth, have small leaves with intervein chlorosis, necrosis, and bud deficiency and root malformations in the vegetation period. There is black discoloration on the woody tissues, as well as dark brown stripes on the rootstocks. Cylandrocarpon species cause longitudinal vascular streaking on young plants.

The Phomopsis dieback was first described by Lehoczy in 1969 in Hungary in a vineyard in Villány. Pycnidia of the pathogens develop on the dead bark, under the bark and on the woody tissues, the matured shoots are becoming white and pycnidia are developing on their surface. The woody tissues have a wedge-shaped brownish discoloration. The diseased plant exhibits reduced shoot growth, dark, humpy, irregular shaped,

brownish lesions on the shoots and the petioles, which longitudinal, drying cracks. The mycelium in green shoots is mainly found in the parenchyma tissue of the bark. Leaves have rounded, irregular, greenish-yellow chlorotic spots, which later become necrotized. The symptoms on the berries are only typical near to the harvest.

The strains, compositions and methods of the present invention can prevent the development of GTDs.

5 Due According to our experimental results, the *Trichoderma* treated grafts did not show the symptoms of GTD later. Following a single application, the *Trichoderma* strains of the invention, unlike other tested *Trichoderma* tested strains, could be re-insolated two years later, consequently, the strains, compositions and methods use of the present invention are able to prevent the development of GTDs for long periods. Less treatment is required by using the strains, compositions and methods of the present invention, and the annual use of *Trichoderma* may
10 become unnecessary. Products containing strains different from *Trichoderma* strains of the present invention, currently authorized in Europe (e.g., Esquive WP - *Trichoderma atroviride* I-1237; Vintec - *Trichoderma atroviride* SC1; Tellus WP - *Trichoderma asperellum* ICC0122 and *Trichoderma gamsii* ICC 080) requires multiple, typically annual treatments for prevention prior to infection.

The strains, compositions and methods of the present invention can be applied at various stages of
15 viticulture. The propagation material (e.g., shoot, grafts, rootstock, and scion) can be effectively treated before the plant is planted, the rootstock and / or the shoot material can be treated prior to the graft preparation, the plant can be treated when a wound is formed (e.g. pruning, injury, and fracture).

An advantage of the strains, compositions and methods of the present invention is that they can be applied over a wide range of temperatures, i.e., treatment is effective at the end of winter, early spring pruning and
20 spring planting. The *Trichoderma* strains according to the invention also grow well at higher temperatures, which is particularly useful in Hungarian conditions. Their effectiveness has been proven in a wide range of varieties and cultivation sites.

The *Trichoderma* strains according to the invention were isolated from the internal woody tissues of the vine, therefore are able to colonize the woody tissues of the treated vine, providing more effective protection
25 against the pathogens.

The *Trichoderma* strains have effectively promoted the growth of treated vines and the development of yields, and are therefore useful as plant growth promoters.

The *Trichoderma* strains efficiently parasitized the fungus of *F. oxysporum*, whose different pathotypes cause diseases of different herbaceous plants (e.g. fusarium wilt of peas, cucumbers, melons, etc.). Based on our
30 *in vitro* results, the strains of the present invention can be used to treat and / or prevent these diseases.

The *Trichoderma* strains can be applied on the plant on (cutting) wounds or other surfaces of the plant (e.g. by brush or spray), e.g. soaking, spraying, or any way appropriate for plant cultivation technology. The strains can be formulated with an agriculturally acceptable carrier and / or additives providing the viability of the spores. Spore compositions can be prepared in a manner known by the skilled person, for example, as described
35 in the Examples section. Depending on the technology used, the concentration of the cells is e.g. $1-9 \times 10^7$ cells / ml, $1-9 \times 10^6$ cells / ml or 10^9-10^5 cells / ml.

Other aspects of the invention are described in the following numbered paragraphs.

1. A method for preventing and / or treating GTD, comprising contacting a grapevine plant or propagation

material of a grapevine plant with at least one *Trichoderma* strain or with a composition comprising at least one *Trichoderma* strain, wherein the at least one *Trichoderma* strain

- is selected from the strains belonging to species comprised in the group of *Trichoderma harzianum* species group, *Trichoderma viride*,

5 - is capable of long lasting colonization a woody tissue of the grapevine plant such, that it can be isolated from the woody tissue more than six months after, preferably more than one year after, most preferably more than two years after said contacting,

- forms appressorium against *Diplodia seriata* and *Neofusicoccum parvum* and has a Biocontrol_index (BCI) value of at least 90%, preferably at least 95%, most preferably 100%..

10 2. Preferably, the *Trichoderma* strain can be detected in a woody tissue of the grapevine plant at least 5 centimeters, preferably at least 10 centimeters from the site of contacting less than 12 months after, preferably less than eight months after, preferably less than six months after said contacting, particularly preferably in any woody part of the grapevine.

15 3. Preferably, the *Trichoderma* strain forms slightly elongated spheroidal conidia in scattered or concentric circles when cultured on potato dextrose agar medium.

4. Preferably, the *Trichoderma* strain can grow on potato dextrose agar medium at about 5 ° C with an average mycelial growth of at least 0.5 mm / day, preferably at least 0.75 mm / day, particularly preferably at least 1.25 mm / day.

5. Preferably, the *Trichoderma* strain belongs to *Trichoderma harzianum* group.

20 6. Preferably, the *Trichoderma* strain forms an appressorium against *Fusarium oxysporum* and has a BCI value of at least 50%, preferably at least 75%, particularly preferably 100%.

25 7. Preferably, the *Trichoderma* strain is a DE_TR04 strain deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession number NCAIM (P) F 001456 or derivative thereof, DE_TR05 deposited under accession number NCAIM (P) F 001457 or derivative thereof.

8. Preferably, the *Trichoderma* strain is DE_TR08 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession number NCAIM (P) F 001458 or derivative thereof.

30 9. Preferably the method comprises contacting the grapevine plant or grapevine propagation material with at least two *Trichoderma* strains, wherein the at least two *Trichoderma* strains are selected from DE_TR04 or a derivative thereof and DE_TR05 or a derivative thereof.

10. Preferably the *Trichoderma* strain or derivative thereof or the composition comprising the *Trichoderma* strain or derivative thereof is contacted with a grapevine plant or propagation material of a grapevine plant showing a symptom of a GTD.

35 11. Preferably, the GTD is black dead arm.

12. Preferably, the GTD is connected to an infection caused by *Diplodia seriata* and / or by *Neofusicoccum parvum*.

13. Preferably the method comprises contacting the plant or propagation material with a *Trichoderma*

strain as defined in any one of paragraphs 1 to 8, or a derivative thereof or with a composition comprising a Trichoderma strain as defined in any one of paragraphs 1 to 8, or a derivative thereof.

14. Preferably, the Trichoderma strain is contacted with the plant or propagation material prior to planting.

5 15. Preferably, the propagation material is folded, grafted, rooted grafts, rootstock, or shoots.

16. Preferably the plant to be treated is Kékfrankos, Cabernet Sauvignon, Cabernet Franc, Hárslevelű, Tar-cal 26 or Furmint grape variety.

17. Preferably, the plant or propagating material is contacted once every two years, preferably every three years, particularly preferably once during the life of the plant with the Trichoderma strain or derivative thereof.

10 18. A method for preventing and / or treating Fusarium oxysporum infection in a plant comprising contacting the plant or a propagation material thereof with a Trichoderma strain as defined in any one of paragraphs 1 to 8, or a derivative thereof or with a composition comprising a Trichoderma strain as defined in any one of paragraphs 1 to 8, or a derivative thereof.

15 19. A Trichoderma strain or derivative thereof, which is a Trichoderma strain as defined in any one of paragraphs 1 to 8, or a derivative thereof.

20. A composition comprising a Trichoderma strain as defined in any one of paragraphs 1-8 or a derivative thereof, and an agriculturally acceptable carrier or additive.

EXAMPLES

20 Isolation of *Trichoderma* sp. from grapevine woody tissues

Our Trichoderma strains were originated from woody tissue of Furmint variety in the Bakonyi vineyard Tokaj Wine Region, Hungary. The strains were isolated from the cordon arm of the healthy trunk that did not show the leaf-symptoms caused by fungi growing in woody tissues (Kovács et al., 2017), although GTD pathogens were detected.

25 The sample preparation and the microbiological tests were done following the method of Kovács et al. (2014c).

Woody parts were debarked and surface sterilized (10% Neomagnol (chlorogen sesquihydrate)) solution than washed twice with sterile distilled water (Kovács et al., (2014c)). After washing the samples wood chips were cut with a sterile scalpel, under aseptic conditions and placed on malt extract agar medium (MEA, Scharlau, Spain) (Crous et al., 2006). Plates were incubated at 25°C temperature for 3 to 5 days, and mycelial fragments from emerging fungal colonies were transferred to new MEA plates to prepare pure cultures. Isolates were maintained on MEA. Mycelial or conidial suspensions were stored on slope agar at 4 °C and in 20% glycerol at -80°C (Kovács et al., 2014c).

Morphological tests

35 The cultures were identified at the level of families and genera based on morphological identification.

Pure fungal cultures were used for the taxonomic identification of the fungi based on colony morphology, characteristics of conidia (color, shape, size), conidiophores, or fruiting bodies (presence, absence) and their characteristics if presented (Kovács et al., 2014b). Based on these informations, the *Trichoderma* strains were separated from other endophytic fungi.

Morphology of *Trichoderma* strains

Conidia were developed in scattered or concentric circles on the medium. (Fig. 1-2) The conidia of our strains were slightly elongated, spherical, with an average diameter of between 2.8 -3.3 μm (Table 1). DE_TR04 grown on PDA medium produced yellow, pigment diffusing into the medium.

5 Table 1.: Morphological characterization of *Trichoderma* strains

Strain code	Species/subclade	Diameter of conidia (SD)	Pattern of conidia formation
DE TR04	<i>Trichoderma harzianum</i> f"lixii"	3.19 μm (0,466)	elszórta
DE TR05	<i>Trichoderma harzianum</i> / II	2.87 μm (0,512)	koncentrikusan
DE TR08	<i>Trichoderma viride</i>	3,34 μm (1,356)	elszórta

Molecular identification of *Trichoderma* strains

Molecular identification were performed based on sequence analysis of the Internal Transcribed Spacer (ITS) region (ITS1, ITS2 (Schoch et al., 2012) of the rDNA, moreover *tef1* and *ech42* gene sequences were used for better identification. Primers used for amplification of the different marker sequences are summarized in

10 Table 2.

Table 2.: Molecular markers used for the PCR amplification of *Trichoderma* strains

Molecular marker	Species	Primer	Sequence	Reference	T* (°C)
ITS1, ITS2	<i>Trichoderma</i> sp.	SR6R	AAGTAGAAGTCGTAACAAGG	White et al., 1990	50
		LR1	GGTTGGTTTCTTTTCCT		
<i>tef1</i>	<i>Trichoderma</i> sp.	EF1 728F	CATCGAGAAGTTCGAGAAGG	Carbone - Kohn, 1999	59
		EF1 986R	TACTTGAAGGAACCCCTTACC		
Chi 18-5 (<i>ech42</i>)	<i>Trichoderma harzianum</i>	Chit42-1a	GCTYTCCATCGGTGGCTGGAC	Kullnig and Gradinger et al., 2002	55
		Chit42-2a	GGAGTTGGGGTAGCTCAGC		

* Anellation temperature

Genomic DNA was extracted with NucleoSpin Plant II (Macherey-Nagel) Kit according to manufacturer's instructions. GoTaq Green Master polymerase (Promega) was used in the PCR reaction. The reaction volume was 25 μL : 12.5 μL Green Master polymerase enzyme (Promega), 0.4-0.4 nmol/ μL of each primer, 12-50 ng template DNA. PCR products were purified with NucleoSpin Gel, PCR Clean Up Kit. DNA sequencing was performed in Microsynth Austria GmbH.

The bioinformatics analysis of the sequences was performed following sequencing. The taxonomic classification of the strains was determined by BLAST analysis of the sequences (Altschul et al., 1990; Kovács et al., 2014a). Sequences were aligned with Clustal-X program (Higgins and Sharp, 1988; Thompson et al., 1997; Larkin et al., 2007) then the GeneDoc program was used for the refinement of the sequences (Nicholas et al., 1997). The exact alignment is necessary for the determination of phylogenetic relationships between the sequences. Phylogenetic trees were created with MEGA 6.0 program (Tamura et al., 2013).

The identification of the *Trichoderma* sp. for the biological protection - isolated from living, asymptomatic woody tissue - first was based on the ITS sequences with comparing the sequences with the deposited GenBank ex-type strains (**Figure 4.**). Maximum Likelihood (ML) analysis was used for drawing the phylogenetic tree. Based on the results, TR01-05, TR 07, TR09-10 strains belong to the *T. harzianum* group, TR06 belongs to *Trichoderma orientale* - *T. longibrachiatum* group and DE_TR08 belongs to the *Trichoderma viride* group. SEQ ID NO: 5, 6 and 7 show the ITS sequences of the strains DE_TR04, DE_TR05 and DE_TR08.

It is necessary to determine the *tefl* (translation elongation factor alpha 1) gene sequence for the exact taxonomical identification of *Trichoderma* strains. Maximum Likelihood (ML) analysis was used for the analysis of the *tefl* sequence like the ITS sequence. Deposited GenBank ex-type strains were also used to create the phylogenetic tree in addition to our strains (Druzhinina et al., 2005). The *tefl* sequence analysis confirmed the result of ITS1 and 2 sequences (**Figure 5**). The *T. harzianum* group includes TR01-05, TR 07, TR09-10, while TR06 is shows greater similarity to *Trichoderma orientale* (teleomorph: *Hypocrea orientalis*). Based on the sequence results, DE_TR05 strain was clearly separated from the most similar *Trichoderma atroviride* strains based on the the *tefl* sequence fragment (SEQ ID No: 3) and arranged in a clade separate with 95% probability (**Figure 21.**). DE_TR04 strain is similar *Trichoderma simmonsii* species based on the *tefl* sequences (**Figure 22.**). **Figure 23.** shows the comparison of the DE_TR04 *tefl* sequence (SEQ ID NO 1) with the most similar *Trichoderma simmonsii* deposited sequences. The consensus sequence is at the bottom line. Gaps are indicated by dash. DE_TR04 strain has a unique nucleotide "T" at position 138th different from the "C" in the consensus sequence at the same position, while at position 156th there is an "A" which it is absent in the deposited sequences. Those differences clearly separates DE_TR04 strain from the deposited sequences of the other strains.

The sequence analysis based on the *GHI8* (glycoside hydrolase 18 gene) chitinase (chi) 18-5 chitinase coding gene is necessary for the precise taxonomic classification of the *Trichoderma* species, in addition the *tefl* gene in case of of *T. harzianum* species (**Figure 5.**) (Druzhinina et al., 2010). Our strains are belong to the following subclasses of *T. harzianum* species complex, based on the *GHI8* sequence analysis: *T. harzianum* sub-clade (TR07, TR10), "H. lixii" subclade (TR01-04), Subclade II (TR05). Based on the *tefl* gene sequence, the TR09 strain is in the Subclade III., while it was grouped within Subclade IV. based on chi18-5 gene. The sequence of SEQ ID No: 2 shows the chi18-5 gene fragment of DE_TR04 strain, the sequence of SEQ ID No: 4 shows the chi18-5 gene section of DE_TR05 strain.

Separation of *Trichoderma* strains with microsatellite fragments

Some of the earlier described microsatellites with described high variance were amplified from our *Trichoderma* strains for a better characterization (identification and differentiation) of following the method described by Rai et al., (2016). The results proved that, our strains can be identified based on the presence of the following microsatellite fragments (Table 3., Figure 26-29.):

		<i>Trichoderma</i> strain ^a		
microsatellite	fragment (bp)	DE_TR04	DE_TR05	DE_TR08
TaSSR1	<100 (A)	+	+	-
TaSSR1	<100 (B)	+	-	-

TaSSR1	<100 (C)	+	+	-
TaSSR1	150	-	-	+
TaSSR1	258	-	+	-
TaSSR1	338	-	+	-
TaSSR1	407	-	+	-
ThSSR1	211	+	+	-
ThSSR1	213	-	-	+
ThSSR6	400	+	-	-
ThSSR6	378	-	-	+
ThSSR6	390	-	+	-
TvSSR1	432	+	+	+
TvSSR5	198	+	+	-
ThSSR4	<100 (A)	-	-	+
ThSSR4	<100 (B)	-	-	+
ThSSR4	159	+	+	+

^a +: amplified fragment; -: no amplification

The amplification conditions are described below.

Segregation of *Trichoderma* strains based on PCR amplification of microsatellites with TaSSR1, ThSSR1, ThSSR6, TvSSR1 and TvSSR5 primers.

5 Materials and methods

PCR primers: TaSSR1 forward 5 'AAGCGGTCAGTTGAAAGTAACG 3'

TaSSR1 reverse 5 'AAGGGTTTTGCTTGTCAGATA 3'

ThSSR1 forward 5 'GCGATTGAGAGGAACGAACT 3'

ThSSR1 reverse 5 'AATCAAGTGAGGATTTGCTGCT 3'

10 ThSSR6 forward 5 'GCGAATGTCACCATCATCTTC 3'

ThSSR6 reverse 5 'TGAGAGAGCCGGAGTATAGGAG 3'

TvSSR1 forward 5 'CTATGGTGCCTCTGGTCTTTTC 3'

TvSSR1 reverse 5 'GATGTTGGACTTGATACCACCC 3'

TVSSR5 forward 5 'GGATCGGCAAGGAATATAAACA 3'

15 TvSSR5 reverse 5 'CAACTTCCATAAAGACCGAAGC 3'

PCR reakció: DreamTaq Green PCR Master Mix (2X) (ThermoFisher Scientific, K1081),

In a MWG Primus PCR device with the following program:

PCR reaction: DreamTaq Green PCR Master Mix (2X) (ThermoFisher Scientific, K1081)

Equipment: MWG Primus PCR with the following cycles:

20 1. Initial (Start) denaturation at 94 ° C for 3 min

Then repeat steps 2-4 five times, reducing the temperature of the anellation (step 3) by 1°C at each cycle:

2. Denaturation 94°C 20 sec

3. Anellation 61 → 56°C 20 sec

4. Polymerization 72°C for 30 sec

Then repeat steps 5-7 36 times

5. Denaturation 94°C 20 sec

6. Anellation 55°C 20 sec

5 7. Polymerization 72°C 20 sec

8. Final polymerization at 72°C for 20 min

Size of fragments was calculated from the image taken by FluorChem M (proteinimple) device, based on values by software from AlphaView SA (Version: 3.4.0; Build: 0729); with TBE buffer, running in 2% agarose gel, EasyLadder I (Bioline, BIO-33046) and with QuickLoad 1 kb (New England Biolabs, N0468S) molecular weight markers.

TaSSR1: DE_TR04: Three fragments below 100 bp (A, B, C)

TaSSR1: DE_TR05: two fragments (A, C) below 100 bp; 258 bp, 338 bp, 407 bp.

TaSSR1: DE_TR08: 150 bp

15 ThSSR1: DE_TR04: 211 bp

ThSSR1 DE_TR05: 211 bp

ThSSR1. DE_TR08: 213 bp

ThSSR6 DE_TR04: 400 bp

ThSSR6 DE_TR05: 390 bp

20 ThSSR6 DE_TR08: 378 bp

TvSSR1 DE_TR04: 432 bp

TvSSR1 DE_TR05: 432 bp

TvSSR1 DE_TR08: 432 bp

TVSSR5 DE_TR04: 198 bp

25 TVSSR5 DE_TR05: 198 bp

TVSSR5 DE_TR08: no visible product

PCR primers: ThSSR4 forward 5 'GTCGTCGGCCATCATTC 3'

ThSSR4 Reverse 5 'TTTCAAGGGCAGGACTCTCTCT 3'

30 As above, except for PCR:

1. Initial (Start) denaturation at 94°C for 3 min

Then repeat steps 2-4 five times, reducing the temperature of the anellation (step 3) by 1°C in each cycle:

2. Denaturation 94°C 20 sec

3. Anellation 62 → 58°C 20 sec

35 4. Polymerization 72°C for 30 sec

Then repeat steps 5-7 36 times

5. Denaturation 94°C 20 sec

6. Anellation 57°C 20 sec

7. Polymerization 72°C 20 sec

8. Final polymerization at 72°C for 20 min

ThSSR4 DE_TR04: 159 bp

ThSSR4 DE_TR05: 159 bp

5 ThSSR4 DE_TR08: two fragments below 100 bp (A, B); 159 bp.

Mycelial growth of *Trichoderma* strains

The mycelial growth rate of the *Trichoderma* strains was examined in 9 cm diameter Petri dish in PDA medium in dark at different temperatures. Variance analysis, Tukey test, non-parametric Kruskal-Wallis test and
10 Dunn's comparative test were used for statistical analysis. The probes were considered significant under 5% P-value. Among the *T. harzianum* strains, the TR01-04 of *H. lixii* subclade and DE_TR05 of Subclade II showed similar growth activity at 18.5-25°C. Their growth optimum was at 30°C, while their growth rate was reduced at 37°C.

Based on the phylogenetic analysis, *T. harzianum* strains (Subclade, **Figure 3-5.**) (TR01 - *H. lixii* sub-
15 clade, DE_TR05 - Subclade II., TR07 - *T. harzianum* subclade, TR09 - Subclade III. Chi18-5 gene section IV) and *T. viride* (DE-TR08) grown slower at the highest tested temperature (37°C) than between 18.5 and 30°C (**Figure 6-8**).

The mycelial growth of *T. orientale* (TR06) was faster than the other two species (*T. harzianum*, *T. viride*). *T. orientale* showed rapid growth at all tested temperatures.

20 The TR01 and DE_TR05 *T. harzianum* strains showed slow growth at 37°C. DE_TR08 *T. viride* and TR09 *T. harzianum* strains also had lower growth activity at the highest temperature compared to the tested strains.

The growth of the strains started at 5°C only after one week. The most intense growth was shown by TR01 (*T. harzianum*, *H. lixii* subclade). TR07 *T. harzianum* subclade and TR10 *T. harzianum* strains showed
25 slow growth (**Figure 10.**).

At 12°C, the growth of the three strains (TR04, DE_TR05, TR06) was tested, which were applied in 2014 as spore suspension for the treatment of symptomatic trunks. DE_TR04 showed intense growth primarily at low temperatures. The TR06 (*T. orientale*) strain showed lower growth rate at 12°C compared to DE_TR04 (*T. har-*
zianum) strain.

Mycoparasitic ability of *Trichoderma* strains

The competition ability of the *Trichoderma* isolates were tested against GTD pathogens (*D. seriata*, *Neofusicoccum parvum*), and *Fusarium oxysporum*.

The tests of mycoparasitic ability of the *Trichoderma* isolates were performed according to the method of Szekeres et al. (2006). (Short description Szekeres et al.: Phytopathogen were inoculated to 9 cm diameter Petri
35 dish, and *Trichoderma* was inoculated 24 hours later, 3 cm from the phytopathogen, with the same method on Petri dish. The site of the inoculation was marked at the bottom of the Petri dish, 1.5 cm away from the center of the Petri dish. When *Trichoderma* is inoculated, particular care should be taken to ensure that the two fungi have the same place. The antagonist process was followed by photographs taken with a fixed Kodak DX 3900 digital camera. The distance (18 cm) was always the same. The original 1800X1200 pixels images were resampled to

half the size of Lanczos filter (Duchon, 1979) using Irfan View v3.95 software. In the digital recordings, the entire area occupied by the colonies of the *Trichoderma* strains and the colonies of *Trichoderma* and the plant pathogen was scanned and measured with Scion Image v4.02 software and drew around by hand. During the analysis, the scale was set to 28,346 pixels per centimeter, ie the unit of computed areas was cm². The antagonistic ability of the *Trichoderma* strains was characterized by the BioControl Index, which calculated according to the following formula:

$$BCI = (\text{area of } Trichoderma \text{ colony} / \text{total area occupied by colonies of } Trichoderma \text{ and pathogenic fungi}) \times 100.$$

The *Trichoderma* sp. and *Diplodia seriata* / *N. parvum* / *F. oxysporum* species were grown on potato dextrose (PDA) medium. Two-days old *Trichoderma* and three days old *Diplodia seriata* / *N. parvum* / *F. oxysporum* isolates were used for the experiments. First *Diplodia seriata* / *N. parvum* / *F. oxysporum* was inoculated to malt agar medium 1.5 cm from the center of the Petri dish, and 24 hours later the *Trichoderma* mycelium was inoculated 3 cm from the pathogen. 10 days later photo was taken from the culture. The Biocontrol Index (BCI) was determined after 10 days.

Variance analysis, Tukey test, non-parametric Kruskal-Wallis test and Dunn's comparative test were used for statistical analysis. The probes were considered significant under 5% P-value.

GraphPad Prism 3.02 (Motulsky, 1999) statistical programs were used to evaluate the results. Each tested strain had Biocontrol Index (BCI) of 100%, so the *Trichoderma* isolates overgrew the pathogens. The devastating effect of the mycoparasite was observed for all examined strains (Figure 11-12.).

T. harzianum coiled around the hyphae of the plant pathogen (*D. seriata*, *N. parvum*), formed appressorium, produces cell wall degrading enzymes and destroying pathogenic fungi (Kotze et al., 2011).

Field test of *Trichoderma* spore solution

Preparation of *Trichoderma* spore solution

The method of John et al., 2005 was followed for the preparation of the concentrated spore suspension. Seven days old culture of three selected strains were used in the field experiment. The strains were grown in Petri dish. 2 ml of sterile distilled water was pipetted into each Petri dish, and the spores were suspended in water by inoculation loop. Thoma chamber was used to check the concentration of the spores.

I. Field experiment: Shoot Treatment in Bakonyi vineyard (2014) (Tarcál)

The first experiment was set up in the experimental plantations of the Research Institute for Viticulture and Oenology, Tokaj Wine region on 06th May 2014. The spore suspension was 107 cells / ml (Figure 11). The TR04, DE_TR05 (both *T. harzianum* species group) and TR06 (*T. orientale*) were choosen for the preparation of the spore suspension, based on their different growth characteristics at different temperatures as well as the mycoparasitic activity against pathogenic pathogens.

The spore suspension was sprayed once or twice on the surface of the fresh pruning wounds depending on the surface area. Previously, *Trichoderma* species were not detected from the treated trunks. The trunks showed GTD symptom in the previous year, and *D. seriata* GTD pathogen was also isolated from the cordon arm.

The treated trunks were checked monthly (photographed and examined the presence of the disease). Samples were collected after three months later, moreover one and two years following the treatment.

The treatment of the older plantations was performed in May 2014 with DE_TR04, DE_TR05, TR06 strains. Three months and one or two years later, the *Trichoderma* strains were re-isolated after the treatment. In 2015, the treated trunk showed more intensive shoot growth than the untreated trunk, and difference in the size of the clusters was also detected. DE_TR04 and DE_TR05 (*T. harzianum* species group) strains were re-isolated from the trunk. The re-isolated *Trichoderma* strains were labelled as TRI01, TRI03 and TRI04. TRI01 isolate showed 96% homology with the used DE_TR05 strain. The TRI03 and TRI04 isolates showed 98% homology with the extype strain (Figure 13.).

Table 3 shows the first results of our field experiment in 2014.

Table 3: Results of *Trichoderma* field experiment (1-3 months)

Examined trunk	1 month	2 months	3 months
1	visible leaf symptom	shoot death	shoot death
2	visible leaf symptom	reduction of leaf symptom	
3	no leaf symptom		
4	reduced shoot growth, no leaf symptom		
5	no leaf symptom		
6	shoot number growth, no leaf symptom		
7			
8	reduced leaf symptom, shoot growth		
9			
10	no leaf symptom		

Four of the treated trunks decreased not only the visible symptoms, but we were able to re-isolate the *Trichoderma* strains 3 months later. The disease-specific symptoms decreased on all the other treated samples, except one (III. row 1. trunk). We concluded that this trunk had been destroyed even before the *Trichoderma* application.

II. Field experiment: Soaking of the pre-plant grafts in the Zuhány vineyard (2015)

Based the taxonomic results, the following strains were used in the field experiments: one *T. harzianum* strain, which was used in the previous field experiment (TR05) and two non *T. harzianum*, endophytes, *T. orientale* (TR06) and *T. viride* (TR08). *Trichoderma* strains (TR06, DE_TR05 and DE_TR08) were inoculated into a 600 mm diameter Petri dishes on the 1st and 2nd April 2015. for the experiment started on the 30th April 2015. *Trichoderma* spores were started to wash with sterile distilled water on the 13th and 14th April 2015. A total of 21 litres of suspensions were collected with a concentration of 10⁹ cells / ml without dilution. For dilution, sterile distilled water was used. The concentration of the spore suspension after the dilution was 10⁶.

The experiment was set in the Villány wine region, in the Zuhány vineyard of Attila Nagy, with nearly 12,000 plants. The grafts were soaked in 300 batches overnight. Laboratory tests have proved the presence of GTD pathogens (prior to the treatment) in case of several grafts, but not all of the grafts were tested due to their

large number.

1 year old rooted grafts were soaked. The shoot part was covered with paraffin wax, so only the rootstock could be contacted with the Trichoderma and the fungicide (8-oxyquinoline sulfate) used as the technological control.

5 The fungicide used for the technological control was used at 0.2% concentration of (2 ml for 1 liter of rainwater). The grafts were soaked for half an hour in that solution.

Characteristics of the grafts used in the experiments:

Rootstocks:

- 5C

10 • 5BB

Shoots (scions):

- Kékfrankos Kt.1.

- Kékfrankos A4-1

- Cabernet Franc E11

15 • Cabernet Franc I-SV-FV5

- Cabernet Sauvignon 337

20 In case of the treatment with Trichoderma spore suspension, 12- to 24-hour soaking was set, except for Kékfrankos KT1, where 24-36 hours of soaking took place due to prolonged planting. The amount of rainwater was 800 liters. Altogether 3,000 grafts were soaked. 1,500 plants were treated with Trichoderma suspensions, while 1,500 were used as control, of which 750 were standard technological control and treated with 8-oxyquinoline sulfate (5 ml per 1 liter) fungicide, and the other 750 were soaked in water. The different varieties were soaked separately.

Grafts were planted with using hydro drill one day following the soaking.

25 III. Field experiment: use of Trichoderma spore suspension in graft production (2015)

425 mL of a Trichoderma spore suspension containing of 10^8 cells / mL was prepared from DE_TR05; TR06; DE_TR08 strains at 25th March 2015, and stored in a refrigerator until 26th March 2015.

A test was carried out under standard conditions to model the soaking volume necessary for 14 rootstocks, grafting shoots, and 7-7 rootstocks-grafting shoots.

30 The amount of liquid was determined by excess so that the canes and pins were always covered with the soaking liquid.

Based on this, we set up the experiment as follows:

- Solution volume of 14 pieces rootstock shoots: 2000 ml

- Solution volume of 14 pieces scion shoots: 600 ml

35 • Solution volume of 7 pieces rootstock shoots: 1000 ml

- Solution volume of 7 pieces shoots (scion): 300 ml

Based on this, the amount of Trichoderma suspension used to treat the rootstock and scion is 15,600 ml.

Prior to the preparation of the solution, buds and tendrils were removed from the nodes of the rootstocks with a disinfected knife.

Subsequently, rootstocks were divided (28-28 plants) into groups with different soaking time (12-hour, 24-hour, 36-hour, and 48-hour treatments).

The rootstocks (all tested 125AA) were used according to their soaking time. First, rootstocks with 48 hours soaking time were further divided into 14 treated, 7 treated controls and 7 standard control groups.

5 Afterwards, scions with appropriate widths (for testing H1104) were chosen from the cutted shoots. Accurate pairing was recorded, which became definitive at the time of grafting, based on the decision of the mating person. GTDs symptoms were detectable on the scions, while pathogens could not be detected from the rootstocks.

10 The mass of rootstocks and scions to be grafted was weighed before soaking. The 15.600 ml solution to be used was prepared from a mixture of distilled water (15.180 ml) and *Trichoderma* suspension (420 ml) in a suitable large glass jar. The mixing was carried out with vigorous shaking and stirring. Subsequently, the first 48 hours of soaking solution was measured: 2000 ml for 14 rootstocks, 600 ml for 14 scions, 1000 ml for 7 rootstock (standard control) and 300 ml for 7 scions, then the substances to be grafted were placed in trays with the appropriate markings and pouring them into the appropriate amount of solution. At the standard untreated
15 control, the same amount of distilled water was used for the infusions. The amount so released completely covered the materials to be grafted.

The pouring process was repeated every 12 hours (soaking for 36 hours, soaking for 24 hours, soaking for 12 hours), so the materials were available at the same time with only a minimal difference in time.

20 Rootstocks under standard treatment or scions were treated with fungicides two hours before the end of the soaking period. For this, 8-oxyquinoline sulphate (5 ml of agent = 0.5% concentration) was used. Before and after soaking, the grafts were weighted and a hydration percentage table was prepared. In addition, we also measured the root length.

25 Conclusively, on the basis of the initial and final weight (after soaking) the water intake was not significantly affected by either the *Trichoderma* spore suspension or by the pure water. Therefore the *Trichoderma* did not increase significantly the water uptake.

The soaked scions and rootstocks were then packaged and labelled according to treatment, and then delivered to the site of the grafting, István Értékes, graft producer in Legyesbénye.

30 After grafting, the grafts were placed in paraffin at 70 ° C to close the site of callus formation. Subsequently, the grafts were immersed in a beta-indole acetic acid powder that promotes rooting. Then, the grafts were prepared for storage separately with sawdust and plastic sheet.

The grafting took place between 12th and 15th May 2015.

Three months after grafting, the main shoots length and the diameter were measured below the node at the 10th internode.

IV. Field experiment: *Trichoderma* treatment in Szálka (2017)

35 The experiment was set between 02nd and 4th May 2017 in Lajvér Winery plantation, Szálka, Hungary. Only two strains of *T. harzianum* species were used for this treatment. The TR06 were excluded from further field experiments because expressed high growth intensity at 37° C, and members of the *T. longibrachiatum* group have been detected as common human pathogens (Sandoval-Denis et al., 2014). The spore concentrate of

DE_TR04 ($\sim 2 \times 10^7$ cells / ml) and DE_TR05 ($\sim 7 \times 10^7$ cells / ml) strains were diluted with well water. After soaking for 30-40 hours, the grafts were planted on the specified terrace. 1.510 treated grafts were installed. The concentration of the spore suspension was 9×10^7 cells / ml. 300 grafts were soaked per vessel. After soaking, some of the grafts were transferred to the laboratory of University of Debrecen under chilled conditions, where, following surface disinfection of the samples, *D. seriata* GTD (prior to treatment) was successfully isolated from internal woody tissues by laboratory tests.

Results of field experiments

IV. Open field experiment: Trichoderma treatment of Szálka (2017): Isolation of Trichoderma species from the Szekszárd Wine Region, after soaking before planting.

After the planting, in the first week of October, 4 plants soaked in Trichoderma solutions were collected for the purpose of re-isolating the Trichoderma (Figure 17). Samples were taken for isolation from approximately every 5 cm of the received plants. It was concluded, that both Trichoderma strains used in the treatment were re-isolated from the plant based on colony morphology. *Trichoderma* could be detected not only from the treated root but also from all the woody parts along the young plant, as far as 20 cm from the roots, which were soaked in *Trichoderma* solution (Fig. 18, the location of samples 1-4 about 5 cm apart). marked).

Trichoderma strains used in the Szekszárd (2017) experiment were able to be re-isolated from several treated plants, and according to colony morphology they were the same like DE_TR04 and DE_TR05 strains used. Molecular characterization of isolates is ongoing.

II. Field experiment: Re-isolation of Trichoderma strains from treated Kékfrankos, Cabernet Sauvignon and Cabernet Franc plants from Villány Wine Region

Grafts were planted in approximately 2 hectares in the Villány Wine Region in 2015, with Kékfrankos (A4-1, Kt1), Cabernet Sauvignon (clone 337), Cabernet Franc (N101, I-SV-FV5, E11) varieties. After the planting, the effectiveness of the applied Trichoderma suspension was examined. In the year following the installation, the control and Trichoderma treated plants had a mortality rate below 1% (Figure 19).

GTD symptoms were not detected in the young plantation. The plants treated with the Trichoderma suspension showed more intensive growth compared to the control plants measured in the year following the treatment. (Figure 20).

In 2016, applied Trichoderma species were managed to re-isolate from some of the young plants treated with Trichoderma suspensions. We were able to re-isolate Trichodermas from necrotic and non-necrotic wood tissues of the treated and control plants in case of the Kékfrankos, Cabernet Sauvignon, Cabernet Franc varieties. They identified as *Trichoderma harzianum* and *Trichoderma orientale*. Sequence alignment is shown in Figures 14 and 15.

Regarding the application and comparison with the type strains deposited in the database, the isolates of SZ_03, SZ_07, SZ_06 showed 99% homology with the applied TR06 strains as well as the type strain.

The isolates SZ_01, SZ_02, SZ_05, SZ_08 showed 98% similarity to the applied DE_TR05 strain and 95% and 98% homology to the deposited strain.

I. and III. Field experiments: Monitoring of grafts and older plantations after treatment with *Trichoderma* suspension.

The *Trichoderma* species were re-insolated annually from the Tokaj and Villány wine regions.

In the Tokaj Wine Region, we used an older Hárslevelű plantation in the Bakony vineyard, in the Villányi Wine Region while pre-planting graft treatment was applied.

In these in vivo experiments, it was found that the applied *Trichoderma* strains reduced the typical symptoms of the disease on the vegetative organs, and that the plants had a higher assimilative weight.

5 Our strains isolated from the grape woody tissues were able to colonize plant tissues efficiently.

Treatments with *Trichoderma* Suspension

Table 4 summarizes the data of in vivo experiments with *Trichoderma* strains, the date of the treatments, and the concentrations of applied spore suspensions.

10 Table 4: Field experiments with *Trichoderma* strains

Date of treatment	Location	Method of treatment	Applied strains	<i>Trichoderma</i> species	Concentration of spore suspension
06. 05. 2014	Tokaj	Wound treatment	DE_TR04; DE_TR05; TR06	<i>Trichoderma harzianum</i> , <i>T. orientale</i>	10 ⁷
26.03.2015	Legyesbénye	Grafting	DE_TR05; TR06; DE_TR08	<i>Trichoderma harzianum</i> ; <i>T. orientale</i> , <i>T. viride</i>	10 ⁸
17.04-20.04. 2015	Siklós, Zuhány	Pre-planting graft soaking	DE_TR05; TR06; DE_TR08	<i>Trichoderma harzianum</i> ; <i>T. orientale</i> , <i>T. viride</i>	10 ⁹
02.05-04.05. 2017	Szálka	Pre-planting graft soaking	DE_TR04; DE_TR05	<i>Trichoderma harzianum</i>	10 ⁶

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 gggtcggcgt tggggatcgg ccctgccttg gcggtggccg tctccgaaat acagtggcgg 480
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	gatctcttgg ttctggcatc gatgaagaac gcagcgaaat gcgataagta atgtgaattg	300
	cagaattcag tgaatcatcg aatctttgaa cgcacattgc gcccgccagt attctggcgg	360
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CLAIMS

1. Method for the treatment of grapevine trunk disease (GTD), comprising contacting the grapevine plant with at least one mycoparasitic *Trichoderma* strain, wherein

- 5 - the grapevine plant is contacted through an intact tissue thereof with a strain selected from the group comprising strains belonging to the following species: *Trichoderma harzianum* species group, *Trichoderma viride*,
- to colonize woody tissue of the grapevine plant with the *Trichoderma* strain such that the *Trichoderma* strain is detectable in the woody tissue at least 5 months after, preferably at least eight months after, most preferably at
10 least 12 month after contacting,
- thereby a fitopathogenic fungus causing GTD in the woody tissue of the grapevine plant is controlled by the at least one *Trichoderma* strain,

wherein at least one symptom of GTD is decreased or eliminated in the grapevine plant contacted with the at least one *Trichoderma* strain, three months, preferably two months, most preferably one month after said
15 contacting,

wherein the *Trichoderma* strain forms appressorium against *Diplodia seriata* and/or *Neofusicoccum parvum* and has a Biocontrol-Index (BCI) value of at least 90%, preferably at least 95%, most preferably 100%,

and wherein the *Trichoderma* strain may be prepared by a method comprising the steps of

- (a) providing a tissue sample comprising a mycoparasitic *Trichoderma* strain from an inner woody tissue of a
20 grapevine plant comprising a pathogenic fungus causing GTD, preferably *Diplodia seriata* and/or *Neofusicoccum parvum*, wherein the grapevine plant shows no leaf symptom of GTD,
(b) isolating the *Trichoderma* strain from the tissue sample of (a),
(c) preparing a culture from the isolate of (b).

2. The method according to claim 1, wherein the grapevine plant is contacted with the *Trichoderma* strain
25 through its root,

- to colonize woody tissue of the grapevine plant other than the root with the *Trichoderma* strain such that the *Trichoderma* strain is detectable in the woody tissue other than the root at least 5 months after, preferably at least eight months after, most preferably at least 12 month after said contacting,
- thereby a fitopathogenic fungus causing GTD is controlled by the at least one *Trichoderma* strain in a woody
30 tissue of the grapevine plant, preferably in the root and in a woody tissue different from the root.

3. The method according to claim 1 or 2, wherein the grapevine plant is a rooted grapevine propagating material, which is contacted with the *Trichoderma* strain through the roots by soaking.

4. The method according to claim 2 or 3, wherein the *Trichoderma* strain is detectable in a woody tissue at a distance of at least 15 cm, preferably at least 20 cm from the upper end of the root two to five, preferably
35 three to five months after said contacting.

5. The method according to any one of the preceding claims, wherein the at least one *Trichoderma* strain is selected from DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No.

NCAIM (P) F 001457, DE_TR08 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001458, and derivatives thereof.

6. The method according to claim 5, wherein the at least one *Trichoderma* strain is selected from DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, and derivatives thereof.

7. The method according to any one of the preceding claims, comprising contacting at least two *Trichoderma* strains with the grapevine plant, wherein the at least two *Trichoderma* strains are selected from DE_TR04, DE_TR05, and derivatives thereof.

8. The method according to any one of the preceding claims, wherein the GTD is black dead arm.

9. The method according to any one of the preceding claims, wherein the GTD is a disease associated with *Diplodia seriata* and/or *Neofusicoccum parvum* infection.

10. The method according to any one of the preceding claims, wherein the grapevine plant is contacted with the *Trichoderma* strain once in every two years, preferably every three years, most preferably once during the lifetime of the plant.

11. *Trichoderma* strain, which

- is selected from strains belonging to the species of the group comprising *Trichoderma harzianum* species group, *Trichoderma viride*,
- when contacted with the root of a grapevine plant, is capable to colonize a woody tissue different from the root such that the *Trichoderma* strain is detectable in the woody tissue different from the root at least five months, preferably at least eight months, most preferably at least 12 months from said contacting,
- forms appressorium against *Diplodia seriata* and/or *Neofusicoccum parvum*, and has a Biocontrol-Index (BCI) value of at least 90%, preferably at least 95%, most preferably 100%,

and wherein the *Trichoderma* strain may be prepared by a method comprising the steps of

- (a) providing a tissue sample comprising a mycoparasitic *Trichoderma* strain from an inner woody tissue of a grapevine plant comprising a pathogenic fungus causing GTD, preferably *Diplodia seriata* and/or *Neofusicoccum parvum*, wherein the grapevine plant shows no leaf symptom of GTD,
- (b) isolating the *Trichoderma* strain from the tissue sample of (a),
- (c) preparing a pure *Trichoderma* culture from the isolate of (b).

12. *Trichoderma* strain according to claim 11, wherein the *Trichoderma* strain is detectable in a woody tissue at a distance of at least 15 cm, preferably at least 20 cm from the upper end of the root two to five, preferably three to five months after said contacting.

13. *Trichoderma* strain according to claim 11 or 12, selected from the group consisting of DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001457, DE_TR08 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001458, and derivatives thereof.

14. *Trichoderma* strain, selected from the group consisting of DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, DE_TR08 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001458, and derivatives thereof, preferably selected from the group consisting of DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, and derivatives thereof.

15. Use of a *Trichoderma* strain according to any one of claims 11 to 14 for promoting plant growth.

16. Propagation material of a grapevine plant, wherein the propagational material has been contacted with a *Trichoderma* strain according to any one of claims 11 to 14, thereby woody tissue of the grapevine plant developing from the propagational material is colonized by the *Trichoderma* strain such that the *Trichoderma* strain is detectable in the woody tissue at least 5 months after, preferably at least eight months after, most preferably at least 12 month after contacting, thereby the grapevine plant is resistant to a pathogenic fungus causing GTD in the woody tissue of the grapevine.

17. The propagation material of a grapevine plant according to claim 16, wherein the propagation material is a rooted graft, wherein said contacting is carried out through the root.

18. The propagation material of a grapevine plant according to claim 16 or 17, wherein said contacting is carried out with a *Trichoderma* strain selected from the group consisting of DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, DE_TR08 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001458, and derivatives thereof.

19. Composition comprising a *Trichoderma* strain according to any one of claims 11 to 14 and an agriculturally acceptable carrier or excipient.

20. The composition according to claim 19, comprising a *Trichoderma* strain selected from the group consisting of DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, DE_TR08 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001458, and derivatives thereof.

21. The composition according to claim 20, comprising at least two *Trichoderma* strains selected from the group consisting of DE_TR04 deposited with the National Collection of Agricultural and Industrial Microorganisms on 12 December 2017 under accession No. NCAIM (P) F 001456, DE_TR05 deposited under accession No. NCAIM (P) F 001457, and derivatives thereof.

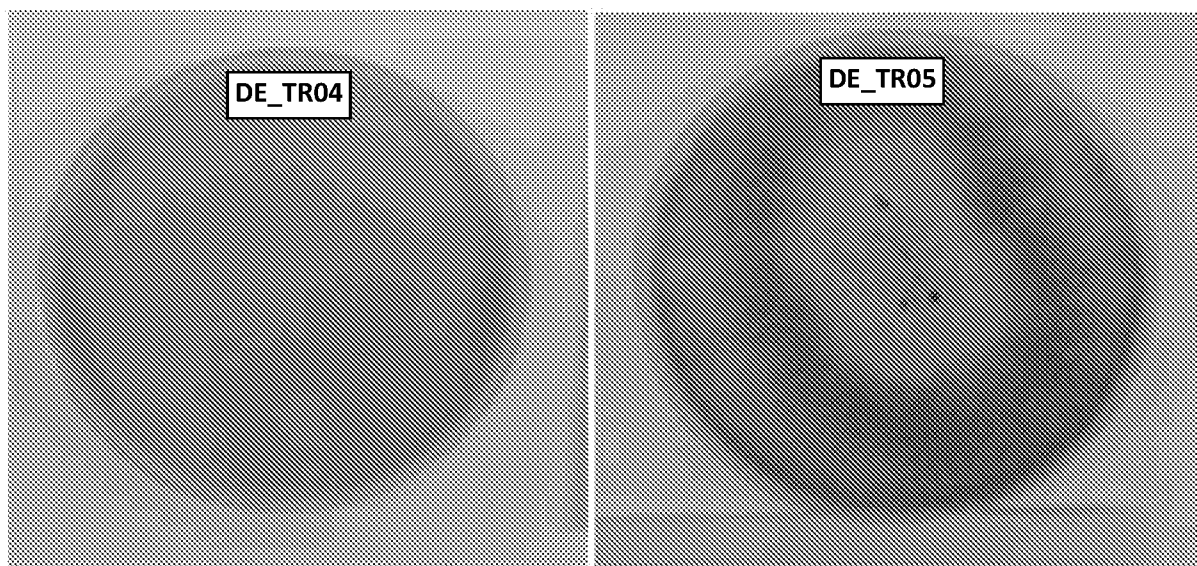


FIG. 1

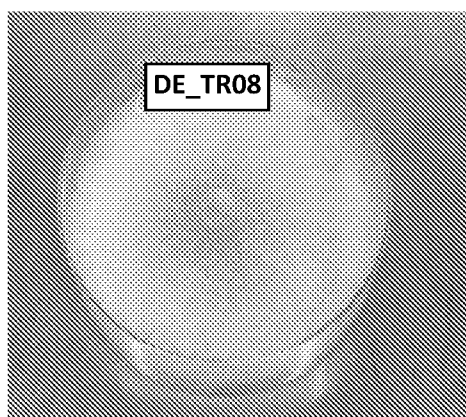


FIG. 2

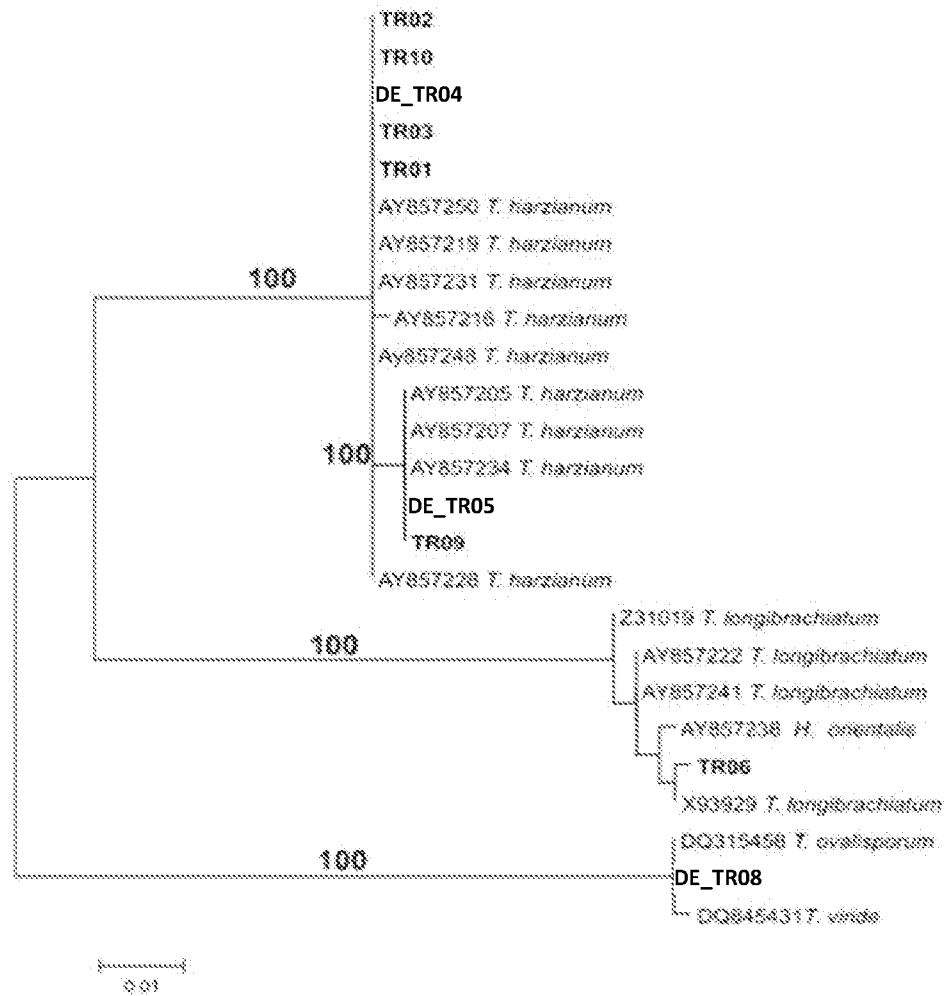


FIG. 3

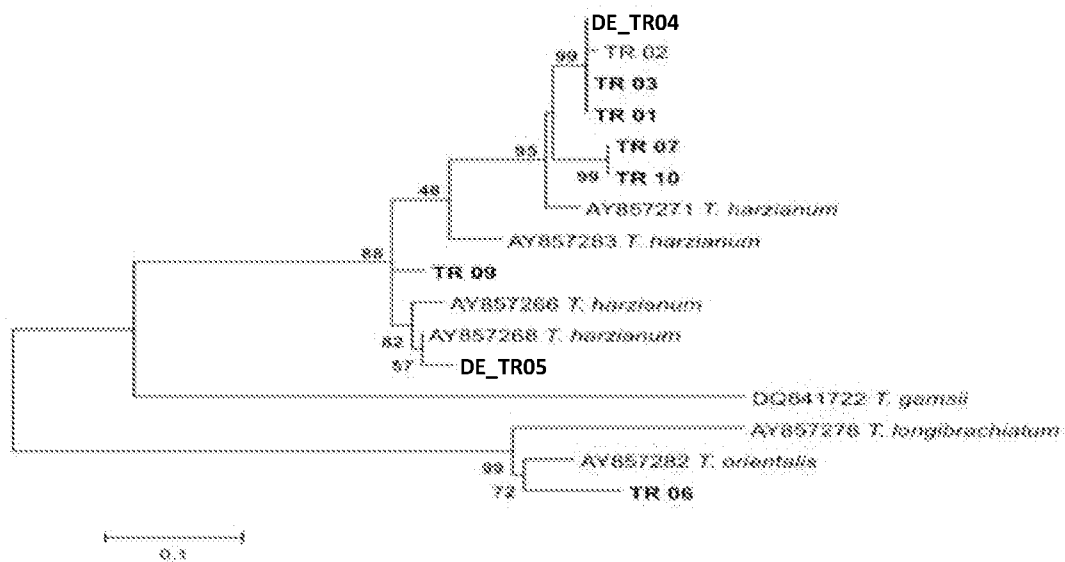


FIG. 4

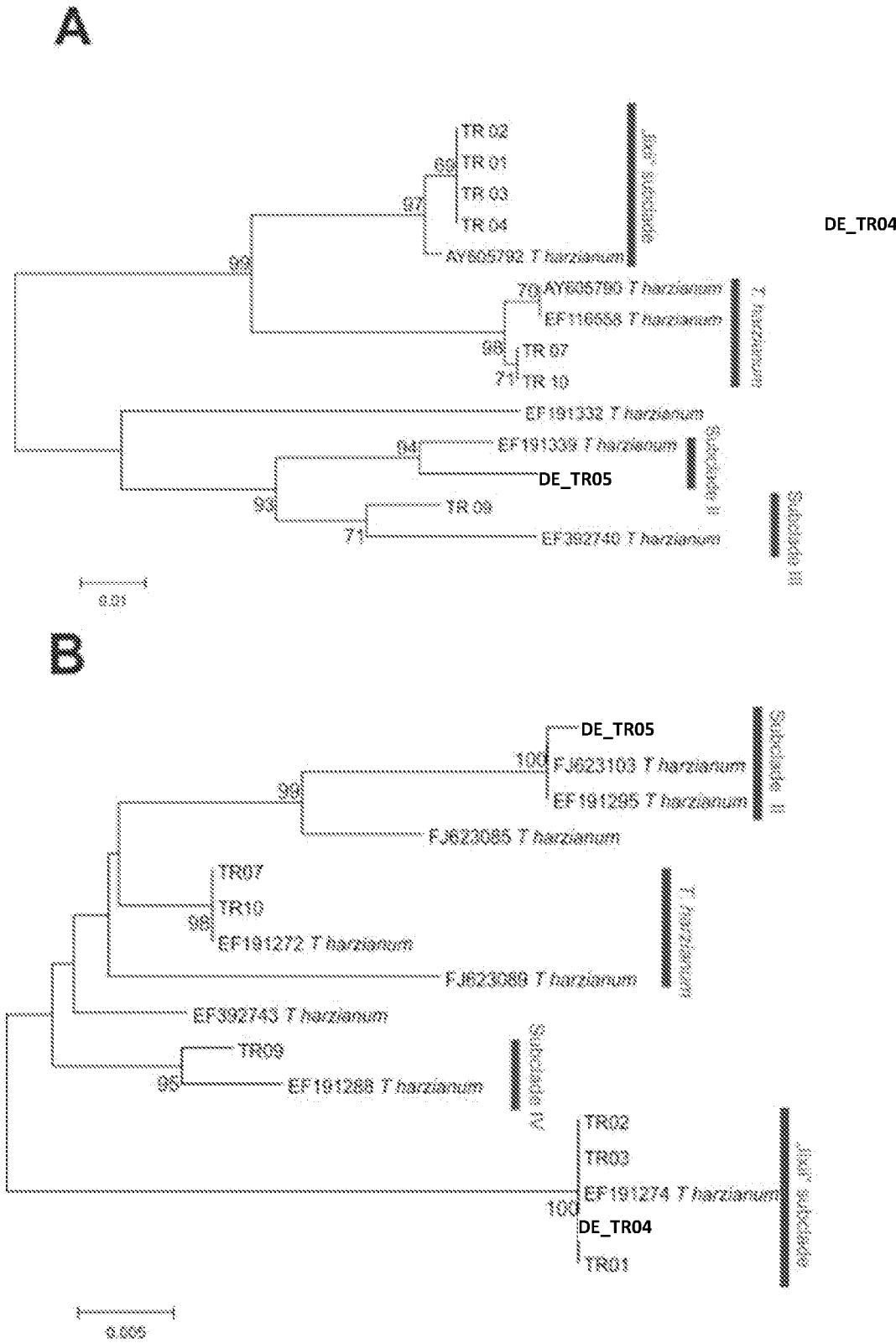


FIG. 5

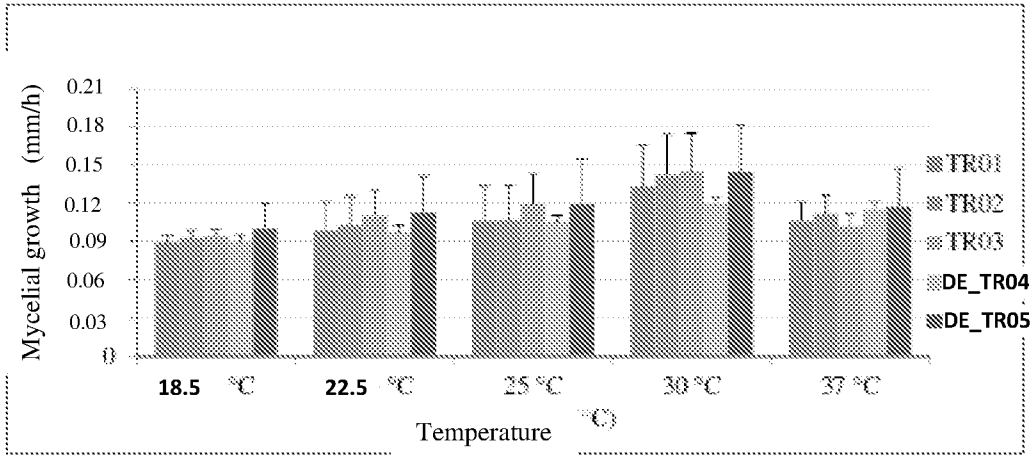


FIG. 6

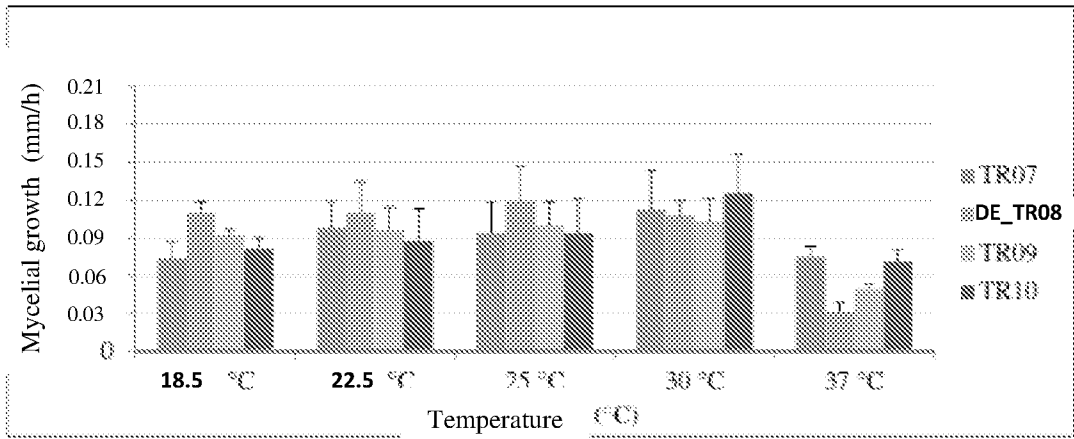


FIG. 7

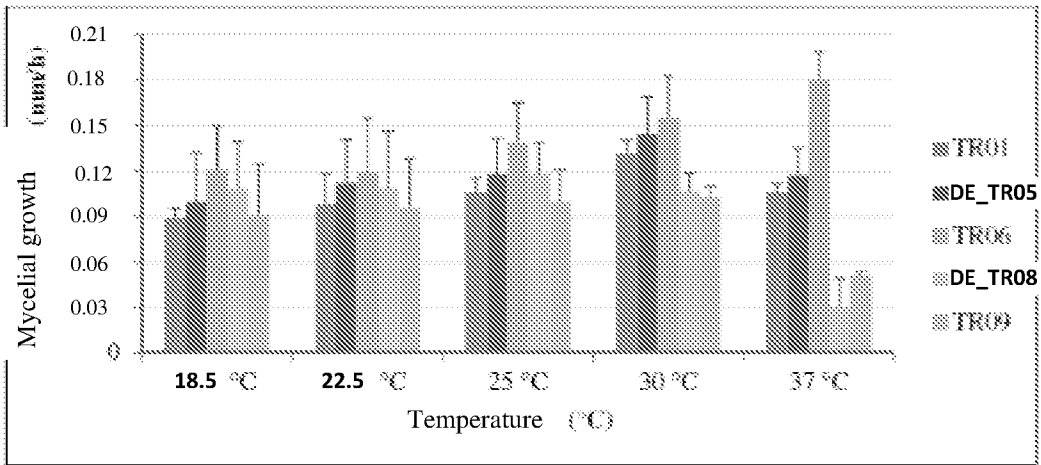


FIG. 8

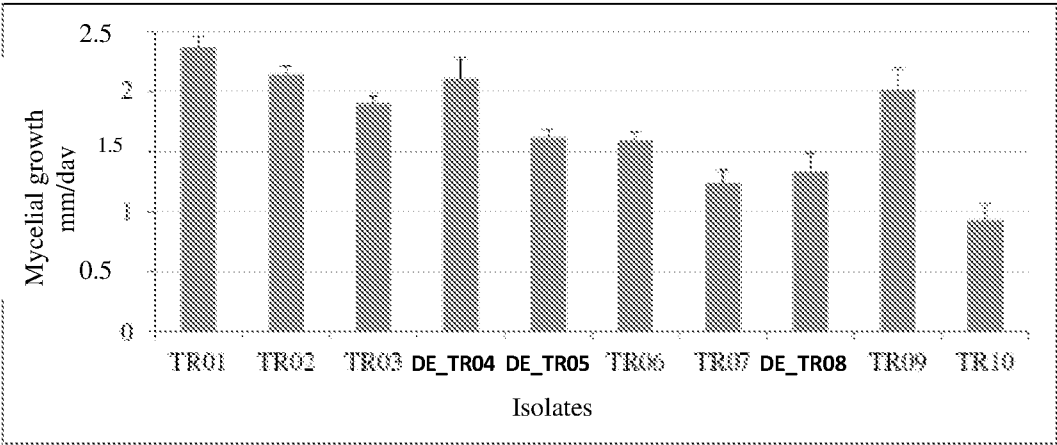


FIG. 9

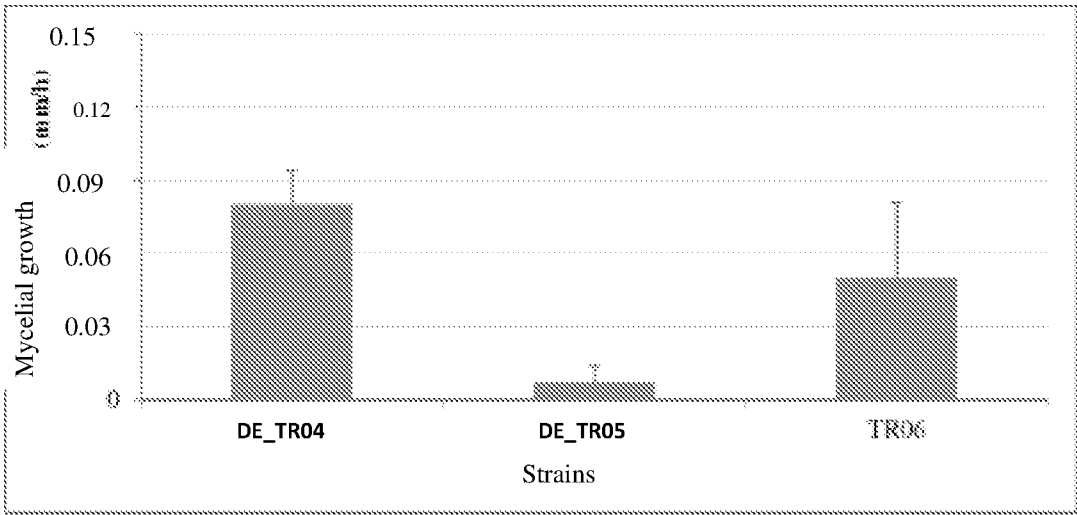


FIG. 10

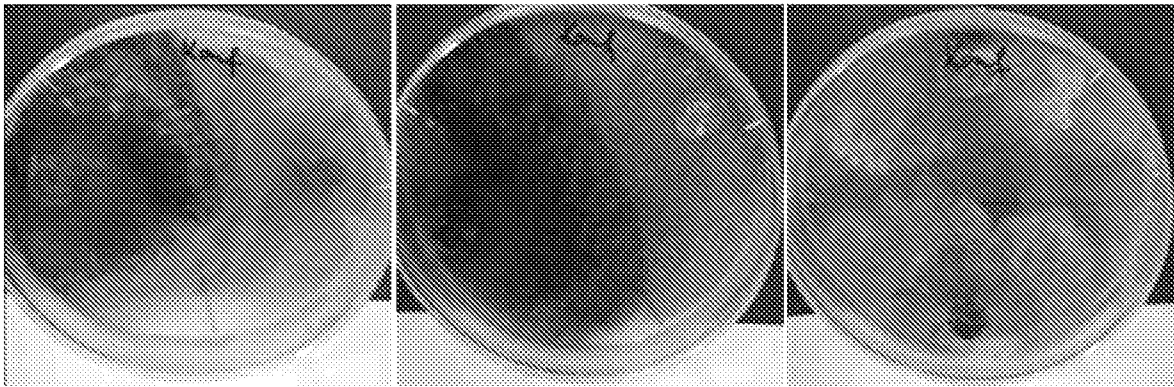


FIG. 11

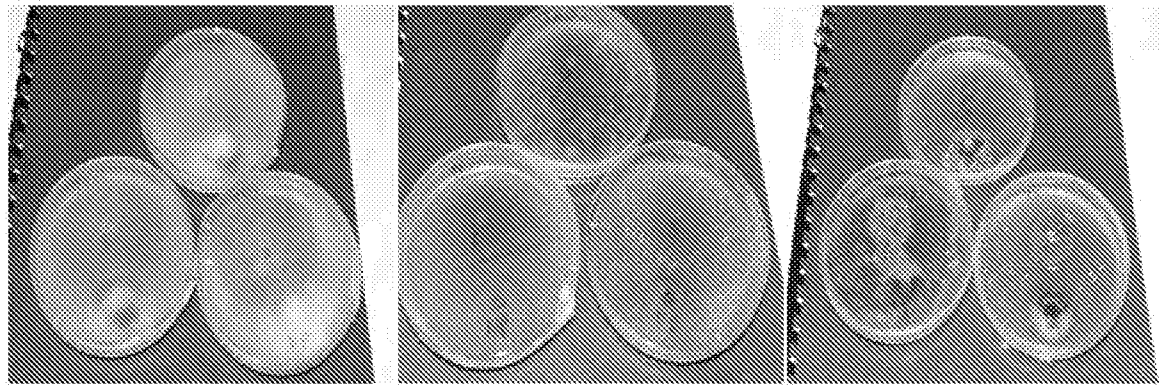


FIG. 12

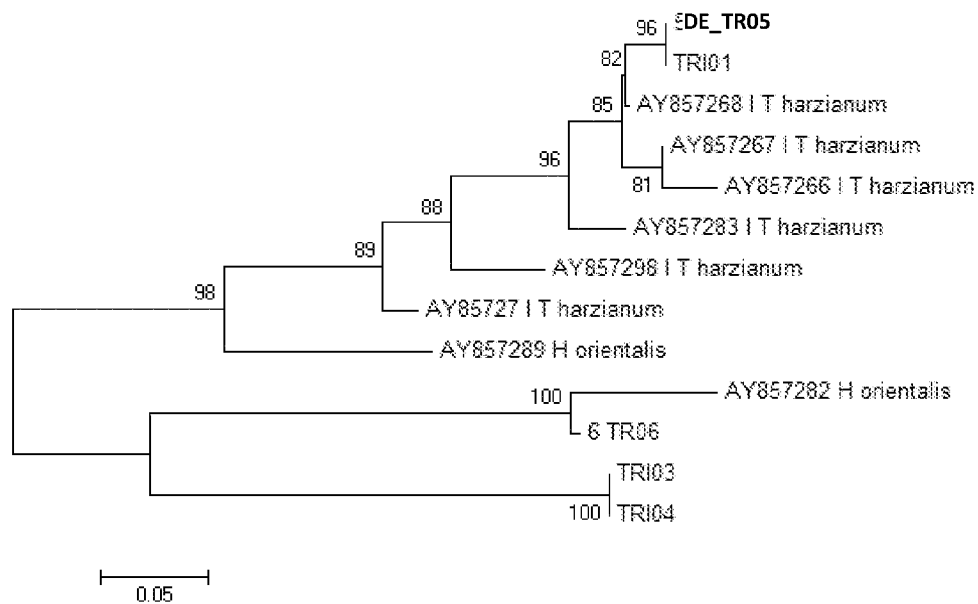


FIG. 13

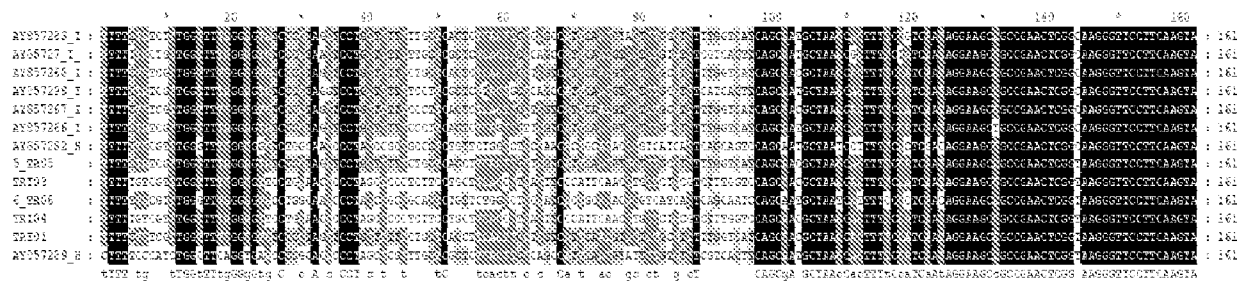


FIG. 14

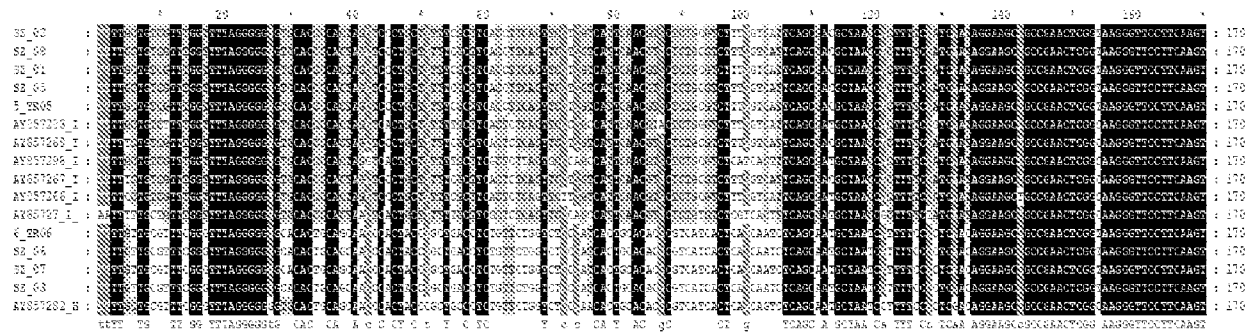


FIG. 15

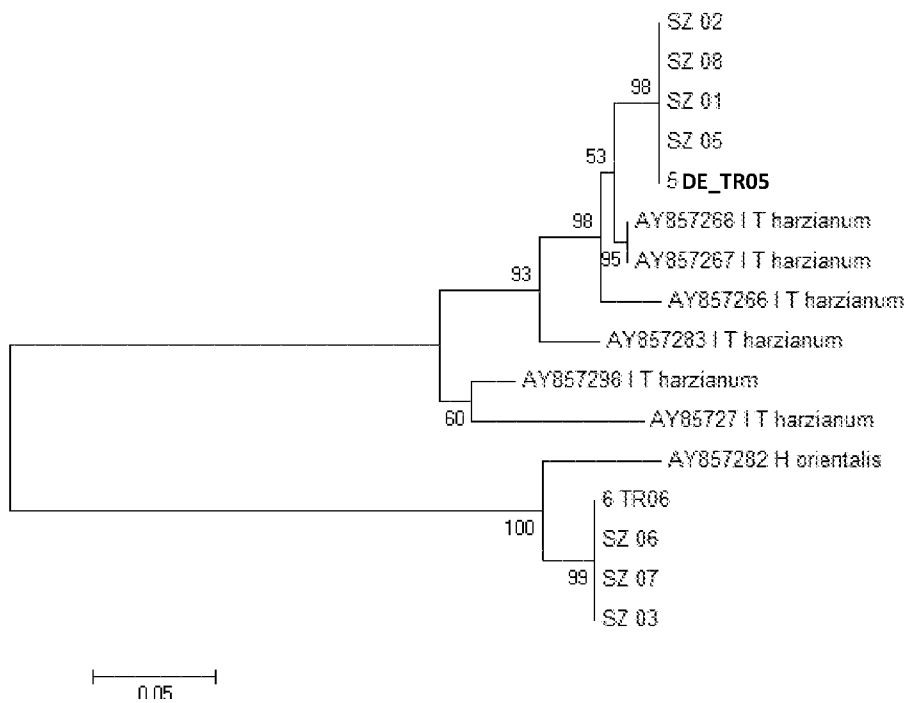
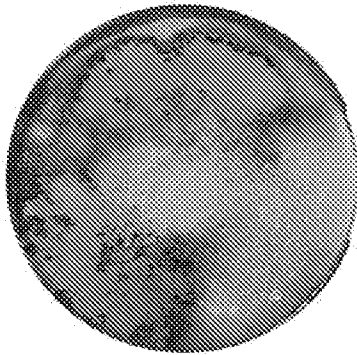


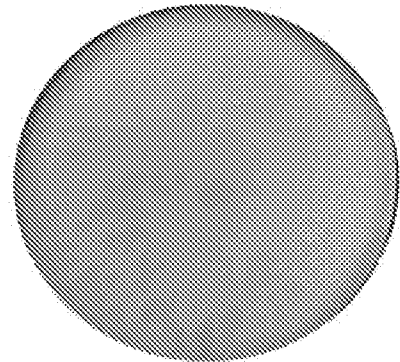
FIG. 16



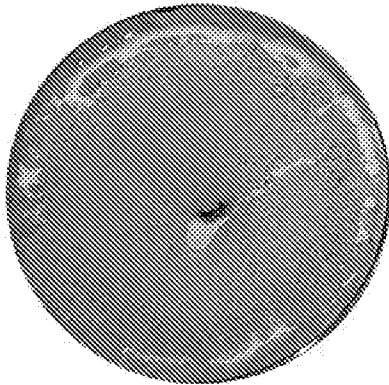
FIG. 17



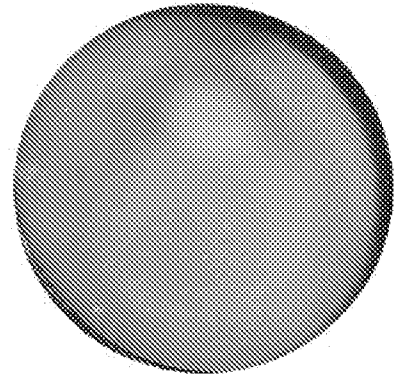
A. T1/1 sample



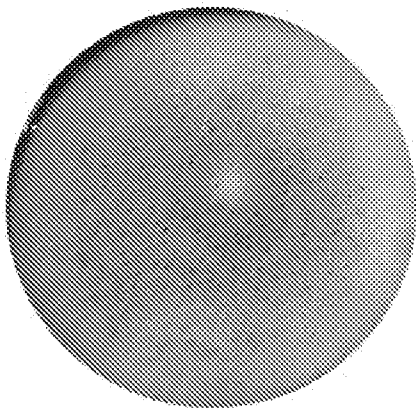
D. T2/5 sample



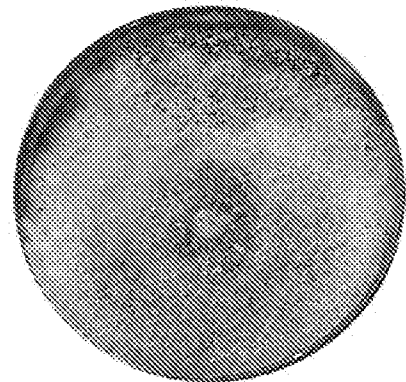
B. T2/2 sample



E. T4/1 sample



C. T2/3 sample



F. T4/3 sample

FIG. 18

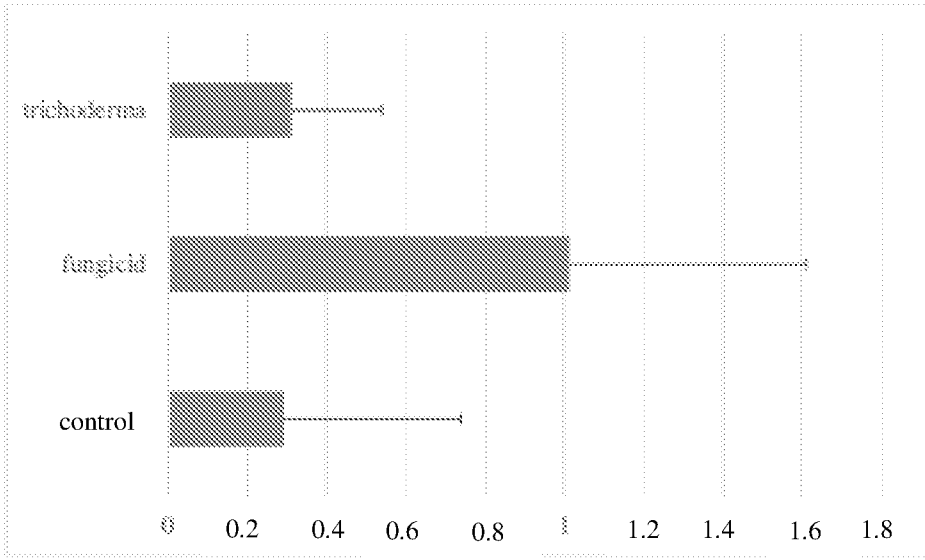


FIG. 19

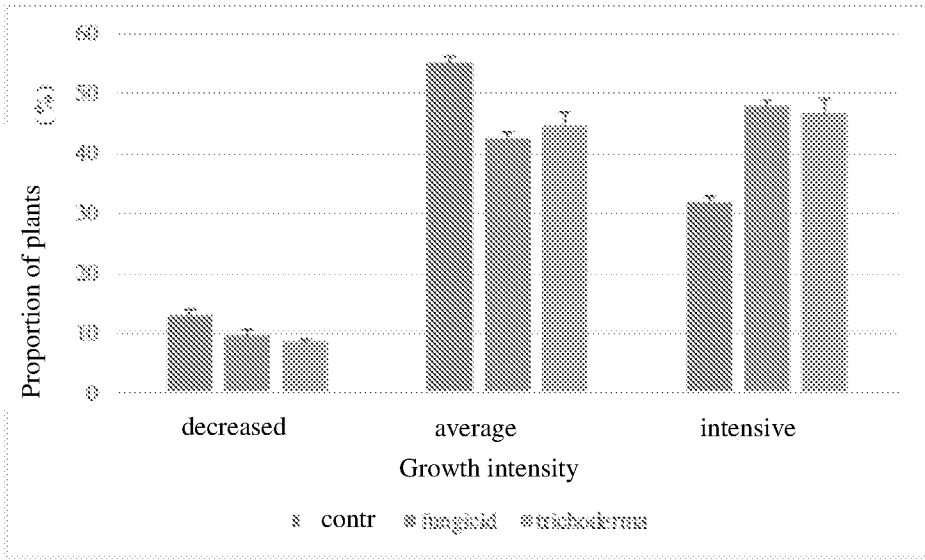


FIG. 20

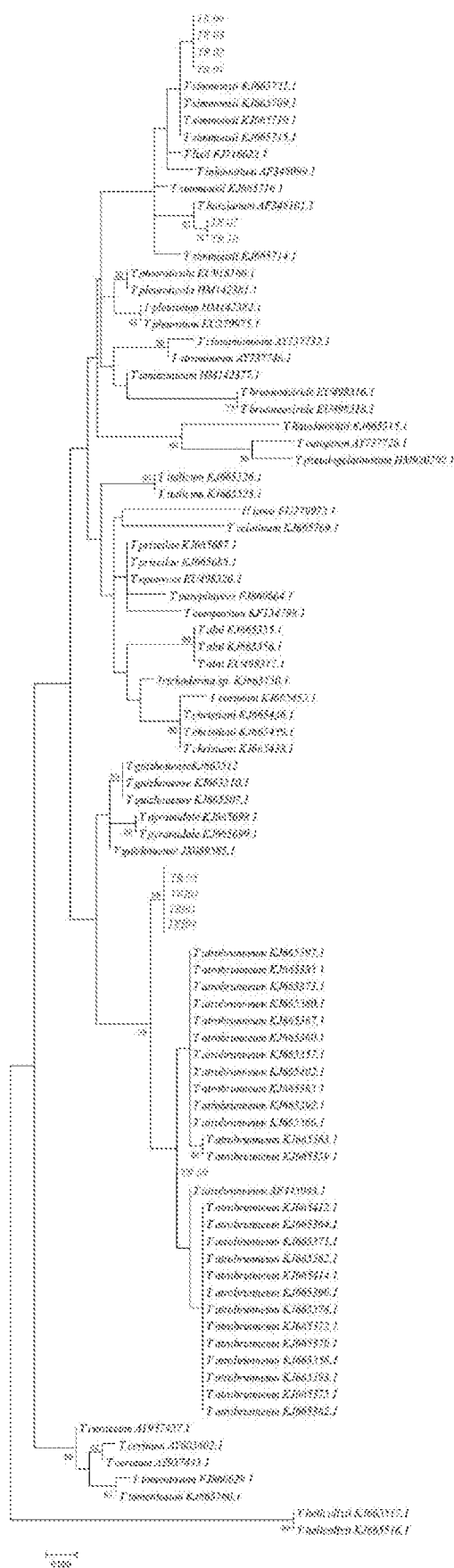
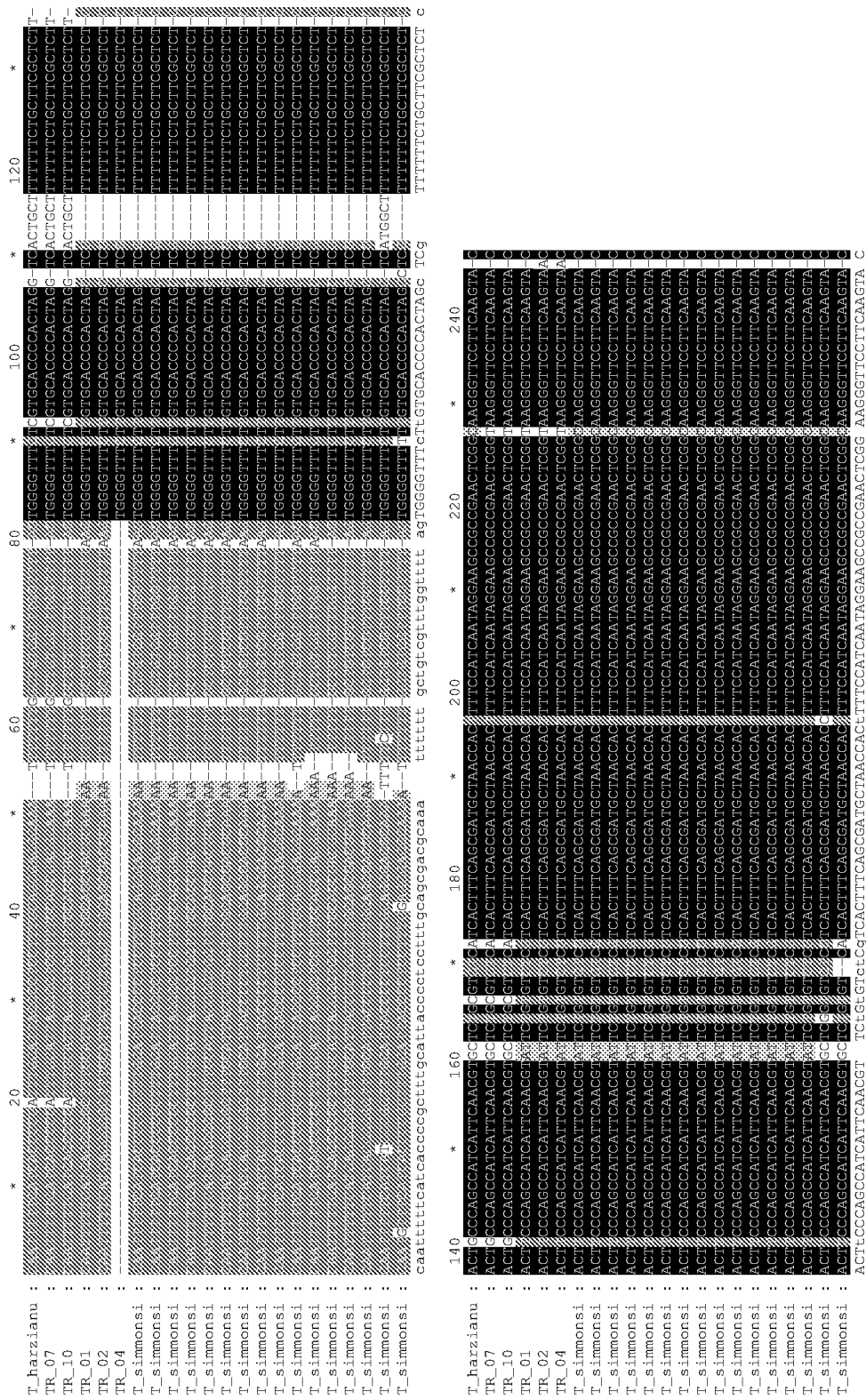


FIG. 21





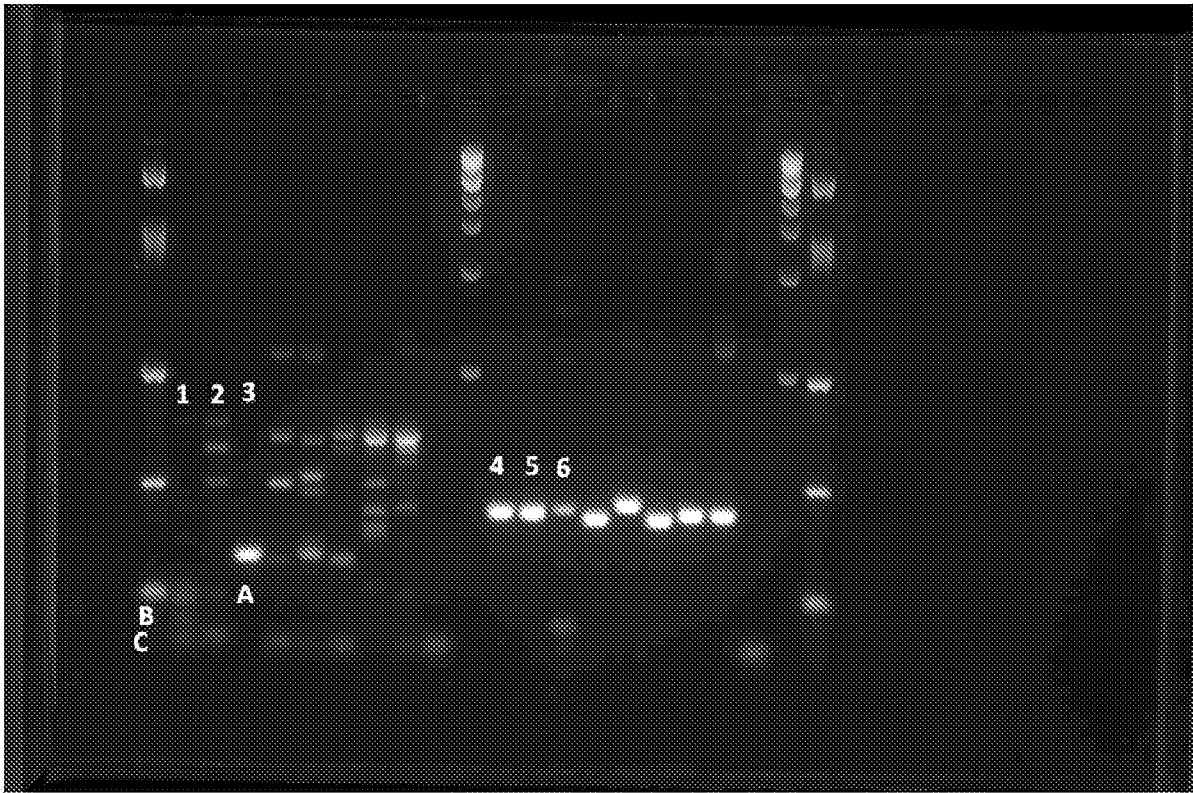


FIG. 24



FIG. 25

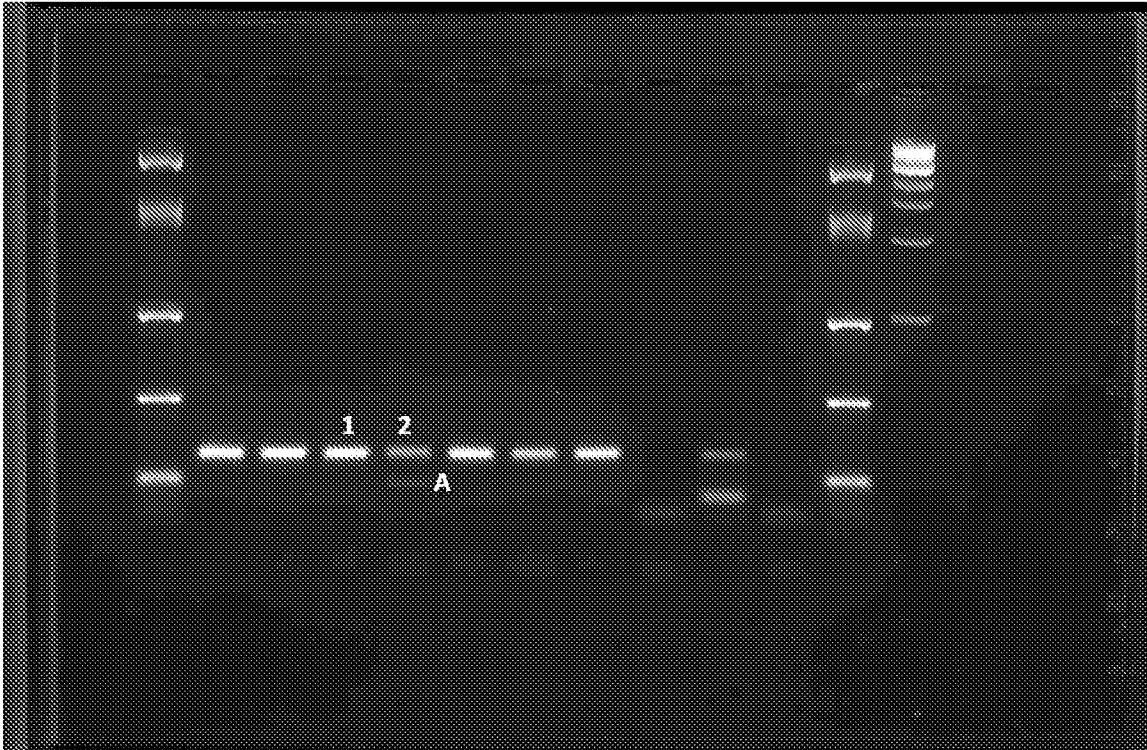


FIG. 26

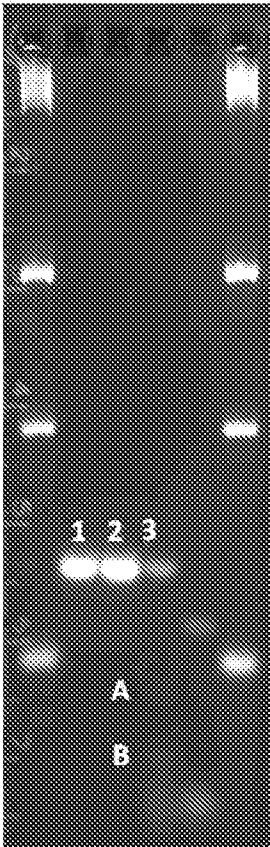


FIG. 27

INTERNATIONAL SEARCH REPORT

International application No
PCT/HU2019/050002

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01N63/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/091337 A1 (UNIV CORNELL [US]; HARMAN GARY E [US]) 12 August 2010 (2010-08-12) claims 1-8,18-27	1-21
X	WO 2005/068609 A1 (B2B SARL [FR]; BESNARD OLIVIER [FR]; BES CLAUDE [FR]) 28 July 2005 (2005-07-28) claims 1-9 page 15, lines 7-28 examples 2,3,6	1-21
X	US 4 678 669 A (RICARD JACQUES J L [SE]) 7 July 1987 (1987-07-07) claims 1-29 table III	1-21
	----- -/-	



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

29 May 2019

Date of mailing of the international search report

11/06/2019

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Authorized officer

Marie, Gérald

INTERNATIONAL SEARCH REPORT

International application No.

PCT/HU2019/050002

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☐ forming part of the international application as filed:
- ☐ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
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2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 363 991 A2 (RICARD JACQUES [SE]) 7 April 1978 (1978-04-07) claims 1-10 example 5	1-21
X	----- DATABASE CAPLUS [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; MUTAWILA, C. ET AL: "Isolation, production and in vitro effects of the major secondary metabolite produced by Trichoderma species used for the control of grapevine trunk diseases", XP002791571, retrieved from STN Database accession no. 2015:2048390 abstract & MUTAWILA, C. ET AL: "Isolation, production and in vitro effects of the major secondary metabolite produced by Trichoderma species used for the control of grapevine trunk diseases", PLANT PATHOLOGY , 65(1), 104-113 CODEN: PLPAAD; ISSN: 0032-0862, 2016, DOI: 10.1111/PPA.12385 10.1111/PPA.12385 -----	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/HU2019/050002

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