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PREPARATION OF COMPOST FROM SEA BUCKTHORN BRANCHES BY USING A MULTIPURPOSE *Trichoderma* STRAIN

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Abstract

This paper presents a process for preparing from sea buckthorn branches sawdust of a compost by using a multipurpose Trichoderma strain (T. harzianum Td50b). The used strain accelerates the degradation of the lignocellulosic material and has biocontrol and plant biostimulant characteristics. The branches, resulting as a by-product of harvesting sea buckthorn berries and leaves, were grounded and extracted to recover bioactive ingredients - flavonols, flavones, phenolic acids, proanthocyanidins, triterpenoids, hydrolysable tannins, serotonin. The resulting sawdust, depleted in polyphenols with potential anti-fungal activity, was normalized to 90% water activity with ammonium nitrate solution 0.2M, supplemented with 10% eggshells powder, and inoculated with 2% alginates beads granules containing 10^9 spores T. harzianum Td50b per gram. After incubation for 50 days at room temperature in aerobic conditions, the survival of Trichoderma was analyzed by cultivation on selective media. The Trichoderma re-isolated from sea buckthorn branch compost maintained its antagonistic and plant biostimulant characteristics.

Key words*: compost, Trichoderma, antagonist, plant biostimulant, sea buckthorn harvested branches.*

INTRODUCTION

The market for products of the sea buckthorn, *Elaeagnus* (synonym *Hippophae*) *rhamnoides* (L.) Nelson, berries, leaves, and derived nutraceuticals, raised significantly in the last years, exceeding USD 2 billion (Janceva et al. 2022). The significant ecological benefits also drive the expansion of the cultivated area with this thorny shrub (Ciesarová *et al.* 2020).

Sea buckthorn berries lack an abscission layer and are harvested biannually, by cutting the whole branches and recovering the berries and leaves after deep freezing. The harvesting process generates a significant amount of by-products. Leaves, rich in antioxidant polyphenols, are used for infusion with health benefits similar to green tea (*Camelia sinensis*) infusions (Cho et al., 2014; Ma et al., 2019). Branches also have a high content of active ingredients - flavonols, flavones, phenolic acids, proanthocyanidins, triterpenoids, hydrolysable tannins, serotonin (Tkacz et al., 2021). The extraction processes valorizing these bioactive components were published (Andersone et al., 2023; Janceva et al., 2022). However, a large amount of extracted material remains after the bioactive ingredients extraction - and new approaches are needed to valorize it.

A possible utilization of the extracted material resulting from sea buckthorn branches is to produce compost, fortified with microorganisms beneficial for sea buckthorn, useful for further improvement of the cultivation (Giurescu et al., 2022). The horticultural functions of the fortified compost are direct, due to nutrients content and

beneficial microorganisms (Sanchez et al. 2017), and indirect/mediated, due to the large quantities of humic acids formed during composting process (Guo et al*.,* 2019) that improve soil structure and properties (Shah et al., 2018), acts as plant biostimulants (Canellas et al., 2015) and support communications between beneficial microorganisms and cultivated plant roots (Shah et al., 2018). Fungi from the *Trichoderma* genera are multipurpose plant-beneficial microorganisms (Woo et al., 2023). *Trichoderma* strains are active against soil-borne pathogens (Dutta et al., 2023; Rodrigues et al., 2023), including *Verticillium* (Kowalska 2021) and *Fusarium* (Ben Amira et al., 2017), fungal pathogens that produce wilt/drying of sea buckthorn shrubs (Drevinska & Moročko-Bičevska, 2021). *Trichoderma* strains are also recognized for their plant biostimulant effects on horticultural crops (López-Bucio et al., 2015). Their application improved flowering, quality traits, and nutrition status in sea buckthorn (Andrzejak & Janowska, 2022).

This paper presents the preparation of a carrier compost for a multipurpose *Trichoderma* strain from our collection (*T. harzianum* Td50b), from extracted sea buckthorn harvested branches. The survival of the *Trichoderma* population, formed from the propagules released from an alginate beads formulation, during the composting process, and the preservation of the antagonistic and plant biostimulant characteristics of *Trichoderma* population from resulted compost were also analyzed.

MATERIALS AND METHODS

Materials

The *T. harzianum* strain Td50b, NCAIM (P) F 001412, is a multipurpose strain. It was proved to have antagonistic activity against soil-borne plant pathogens, mainly determined by the production of volatile bioactive compounds, including 6-PP, 6-pentyl-2H-pyran-2-one (Oancea et al., 2017a; Răut et al., 2014). Td50 strain was also proved to have a plant biostimulant activity on cultivated shrubs (Şesan *et al.* 2020) and to accelerate the degradation of lignocellulosic material (Oancea et al., 2017a). The plant biostimulant effects of (re)isolated *Trichoderma* was tested on *Arabidopsis thaliana*, cv. Columbia (WeberSeeds, Vaals, Netherland). The branches were harvested from sea buckthorn (*E. rhamnoides*, cv. Mărăcineni), organically cultivated in the BioCătina orchard from Valea Marea, Dâmbovița, Romania - 44°46′44″N latitude, 25°14′18″E longitude, 218 m altitude. The BioCătina orchard is established on molic-vertic preluvosol soil, with a high content of coarse sand, slight acidic reaction (pH of 6.4. in water) and a rather low humus content - 3.16% in the upper (0-20 cm) soil horizon. The multiannual average values (1975-2020) of temperature, total precipitation, sunshine daily duration, and wind speed for the BioCătina orchard are 10.1°C, 512.1 mm, 7.1 h and 3.8 $m.s^{-1}$, respectively. The chicken eggshells used as an additive for supplementation with calcium of the extracted sawdust were obtained from the production of the embryonic extract (Hipocrate 2002, Bucharest, Romania). The ingredients for *Trichoderma* cultivation media were supplied by Scharlab (Barcelona, Spain). For *Trichoderma* selective media chloramphenicol, streptomycin, pentachloro-nitrobenzene (quintozene) were supplied by Sigma-Aldrich (Merck Group, Darmstadt). The source for propamacarb was Proplant 72.2 SL fungicide (Adama, Ashdod, Israel). The Murashige and Skoog Basal Medium used to cultivate *A. thaliana*, sodium alginate (30-40 kDa average mass) and calcium chloride used for alginate beads formulation, the reagents for determination of total polyphenols and total flavonoids contents, including gallic acid (≥ 99%) and quercetin (≥ 95%) used as references, the dimethyl formamide used for chlorophyll extraction and the Breathe-Easy® sealing membrane were purchased from Sigma-Aldrich (Merck Group, Darmstadt, Germany). As protective filler for *Trichoderma* spores was used diatomaceous earth (DE) from the Sibiciu quarry (Sibiciu, Pătârlagele, Buzău). This DE was formed as deposit by marine diatoms in Oligocene and contain nanoporous diatom frustules and small amount of clay, illite and kaolinite (Moale et al., 2021).

Sea buckthorn branch extraction

The harvested branches from BioCătina organic orchard were frozen at -39°C in an industrial

freezer for 2 days. The berries and leaves were separated by gentle shaking. The empty branches were cut into small piece by an electric shredder (UD2500, Makita, Aichi, Japan). The cut branch pieces were finely grinded in cryogenic mill (Antylia Scientific, Vernon Hills, IL, USA). The extraction of the resulted saw-dust was intensified by using microwave (Sharma et al., 2008). Portion of 100 g of sawdust were introduced with 1000 mL absolute ethanol in a 2 L flat bottom flask with ground glass joint. The flask was introduced in a microwave extractor Minilabotron 2000 (Sairem, Décines-Charpieu, France). The neck of the flask was taken out from the microwave oven and a condenser feed with cold water was mounted in its ground glass joint. The extraction was done for 12 min, at 1000 W, 2.45 GHz microwave, in the temperature control mode. At the end of the extraction cycle the sawdust was separated from ethanol by filtration and dried in a vacuum oven (VD23, Binder, Tuttlingen. Germany). In ethanolic extract was determined the total polyphenols content (TPC) and total flavonoids content (TFC). The TPC was determined by a miniaturization of the Singleton and Rossi method (Michel et al., 2012), using 96 well plate and reading the absorbance at 765 nm in a microplate reader (CLARIOstar, BMG LabTech, Ortenberg, Germany). The reading were expressed as mg of gallic acid equivalent (GAE) per g of dried weight (d.w.), by using a calibration curve made with gallic acid. The TFC was determined by a miniaturization of the aluminum chloride method (Cacique et al., 2021), using quercetin equivalents (CQ) to transform spectrophotometric readings at 425 nm into flavonoids content.

***Trichoderma* inclusion in the alginate beads**

Td50b strain was cultivated in an optimized medium with the following composition: 34.2 g/L glucose, 0.37 g/L ammonium sulfate, 0.8 g/L yeast extract, 2.7 g/L soymeal, 1.2 g/L K_2HPO_4, and 1.7 g/L KH_2PO_4. This medium was shown to promote chlamydospores formation (Zamfiropol-Cristea et al., 2017). The growing conditions were also chosen to promote strain sporulation - aerated and agitated medium in an orbital shaker (Unimax 2010, Heidolph, Nuremberg, Germany), at 25°C maintained in a transparent hood, with additional light for 16 h per day. The initial inoculation was done with 10^7 spores per ml, recovered from the sporulated culture on PDA medium. The Td50b strain was grown for 2 weeks in the aerated and agitated medium. At the end of the growing cycle, the fungal suspension was aseptically poured through a cotton tissue and the spores were concentrated by centrifugation (Oancea et al., 2016). The alginate beads were produced by coagulation in a 0.25M $CaCl_2$ bath of the droplets resulted from a mixed suspension, 2% DE and 10^8 Td50b spores, in 3% sodium alginate solution (Oancea et al., 2017b). The efficiency and reproducibility of the process was assured by using an equipment designed for beads formation, Encapsulator B-390 (Büchi, Flavil, Switzerland). The beads were dried at 35°C, in a vacuum oven (VD23, Binder, Tuttlingen).

Compost preparation

The resulting dried sawdust, depleted in polyphenols with potential anti-fungal activity, was normalized to 90% water activity with ammonium nitrate solution 0.2M - from 72.3 to 76.9 ml solution per 100 g of different batches of extracted sawdust. The water activity in the sawdust was determined by using a water activity measuring device, LabMaster-aw neo (Novasina, Lachen, Switzerland). The watered sawdust was supplemented with 10% eggshells powder, as a source of calcium and a pH buffering ingredient. The eggshell powder was obtained after washing abundantly with tape water, followed by rinsing with pure water, drying in the vacuum oven and grinding in a planetary ball mill (PM 200 Retsch, Verder Scientific, Haan, Germany), with agate jar and agate balls. The mixture watered sawdust - eggshell was inoculated with 1% alginates beads granules containing 10^8 spores *T. harzianum* Td50b per gram. The inoculated substrate for compost preparation was incubated at room temperature in aerobic condition (Haddadin et al., 2009), using 500 mL round bottom flask with large neck, containing ~250 g composting mixture and fitted with perforated caps and polyethylene tubes for aeration. The aeration with 0.25 L of air/min was done 15 min at every 4 h, using oil-

less air, from a screw compressor (OLS11-YD, Adicomp, Isola Vicentina, Italy). After 50 days of incubation at room temperature in aerobic condition, the compost was analysed for survival of *Trichoderma* and maintenance of the antagonistic and plant biostimulant characteristics. The control was represented by the same composting mixture, saw dust from sea buckthorn extracted for polyphenols, watered with ammonium nitrate solution and mixed with eggshell, without inoculation with alginate beads containing *Trichoderma*. Each experimental treatment, inoculated with alginate beds containing Td50b and control, included 5 jars, aerated at the same level by using an air distributors with mini valves and flowmeter. Each jar, with control pr the inoculated substrates, were weighted initially and after 50 days of incubation, by using an electronic scale.

***Trichoderma* (re)isolation from compost**

The selective medium for *Trichoderma*, containing chloramphenicol, streptomycin, quintozene, and propamocarb was used for re-isolation of *Trichoderma* (Williams et al., 2003). Briefly, a sample of around 10 g compost was mixed vigorously in aseptic condition with 500 ml sterile solution of phosphate buffer in a sterile plastic bags (Intervoid®, Coveris, Vienna, Austria). Serial dilution were performed, and 0.1 mL were platted on *Trichoderma* selective medium. From isolated colony developed after 5 days of incubation at 25°C, samples were taken and observed at microscope for typical Td50b characteristics (Oancea et al., 2017a).

Determination of antagonistic and plant biostimulant characteristic of (re)isolated *Trichoderma*.

The antagonistic activity was tested against *Fusarium graminearum* DSM 4527, by using the dual confrontation assay in a potato-dextrose-agar (PDA) medium (Whipps, 1987). The plant biostimulant assay was done by determining chlorophyll content of *A. thaliana* (cv. Columbia) seedlings exposed to volatiles produced by reisolated *Trichoderma* (Hung et al., 2013). Briefly, *A. thaliana* seeds were germinated and grown for 10 days on Murashike and Skoog (MS) medium, in a 9 cm Petri dish, at 22 ± 2°C with a light/dark photoperiod of 16:8 h (FitoClima 600, Aralab, Rio de Mouro, Portugal). The 10 day old seedling were exposed for 48 hours to volatiles produced by a 3 days old culture of re-isolate *Trichoderma* in a PDA medium distributed in a 9 cm Petri dish. The dish with *Trichoderma* was put together with the dish with *Arabidopsis* and separated by Breathe-Easy® sealing membrane. The incubation of the joint plated was done in the same conditions - at 22 ± 2°C with a light/dark photoperiod of 16:8 h. After 48 h the chlorophyll content was determined after extraction with dimethyl formamide (Zhang & Huang, 2013).

Statistical analysis

The experiments results were submitted to analysis of variance (ANOVA), using the SPSS 21 software package (IBM, Armonk, NY, USA) and Fisher's Least Significant Difference (LSD) test.

RESULTS AND DISCUSSIONS

The cascading process for the preparation from sea buckthorn branches sawdust of compost with the multipurpose *T. harzianum* Td50b stain that we developed in this work is illustrated in Figure 1.

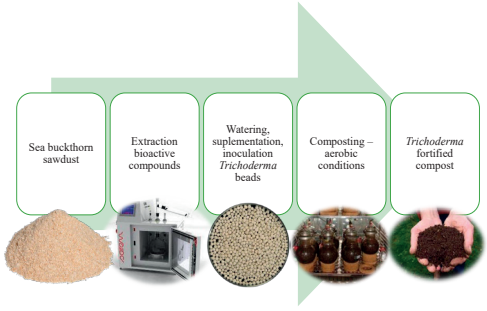

Figure 1. Illustration of the process for compost preparation from sea buckthorn branches by using a multipurpose *Trichoderma* strain

It involve grinding the harvested branches, bioactive extraction from the resulted branches sawdust, watering and supplementation of the extracted sawdust with nitrogen and calcium sources, inoculation with alginate beads containing *Trichoderma* strain and incubation for composting, 50 days in aerobic conditions.

The bioactive compounds were recovered from sea buckthorn branches according to pyramid-value principle, i.e., recovering of high-value components, mainly those useful for wellbeing industries, from biomass, prior others (bio)technological processes (Constantinescu-Aruxandei & Oancea, 2019). The total polyphenols and flavonoids contents is presented in Table 1.

Table 1. The total polyphenols and total flavonoids contents in the microwave assisted extract from sea buckthorn branches (mg/g d.w. extracted sample)

	Total polyphenols content (TPC)	Total flavonoids content (TFC)
Sea buckthorn branches - sawdust produced by cryo-milling with liquid nitrogen	56.8±9.9	48.7±8.2

The obtained values agree with the TPC and TFC contents determined by other authors in the sea buckthorn branches. The resulted extracts are useful for production of cosmetics and/or nutraceuticals, due to their high biological activity (Skalski et al., 2018; Żuchowski, 2023) – and correspond to the above mentioned principle of value pyramid. Also, the extraction of polyphenols with anti-microbial activities (Janceva et al., 2022; Tian et al., 2018) from branches promote the composting process.

The process used for production of Td50b spores and their formulation in alginate beads assure a survival of *Trichoderma* propagules and a good capacity to slowly release these propagules - Figure 2.

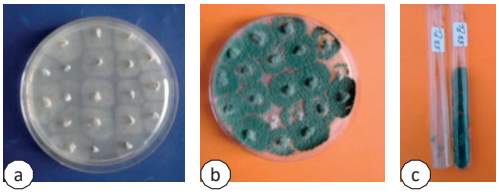

Figure 2. Release of the multipurpose *Trichoderma* Td50b strain from prepared alginate beads: a) immediately after alginate beads preparation; b) *Trichoderma* fungi released during a week of incubation on PDA medium from beads stored 1 month at room temperature; c) *Trichoderma* fungi released during a week of incubation on PDA medium from beads stored 3 month at room temperature

The medium and the cultivation conditions promote the formation of chlamydospores, fungal spreading forms with high resistance to harsh conditions (Zamfiropol-Cristea et al., 2017). Td50b is a strain with high saprophytic competence, able to survive and colonize composting substrate, plant rhizosphere and plant phyllosphere (Oancea et al., 2017a; Oancea et al., 2016; Şesan et al., 2020). The inoculation with *T. harzianum* Td50b alginate beads accelerate the decomposition of the extracted sea buckthorn sawdust by more than 23% - Figure 3.

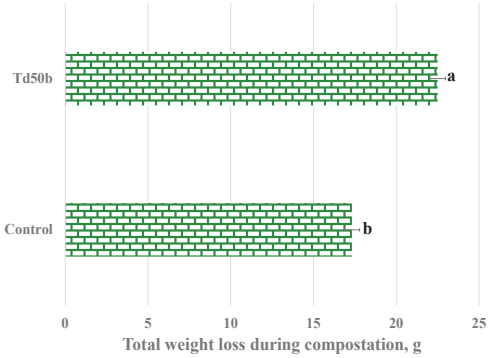

Figure 3. Total loss during the compostation process for the control (un-inoculated with lignocellulolytic microorganisms) and the treatment by inoculation with alginate beads releasing multipurpose *T. harzianum* Td50b strain. Different letters means statistically significant difference ($p<0.05$, $n = 5$)

Td50b strain produce significant amount of proteins acting on the lignocellulose matrix, enzymes - β-glucanases, xylanases, lytic polysaccharide mono-oxygenase (LPMO), laccases, peroxidases, and non-catalytic proteins (cerato-platanins) with expansin-like activities on lignocellulose (Oancea et al., 2016).

The cerato-platanins, non-catalytic proteins, loose the cell wall structure, due to breakage of the physical (hydrogen) bonds that stabilize cellulose and hemicellulose fibrils and promote the activity of enzymes acting on cellulose, hemicelluloses and lignin (Pennacchio et al., 2021). The enzymatic cocktails produced by Td50b strain release oligosaccharides and oxidize lignin, further enhancing composting process (Wu et al., 2022).

The rate of survival of the inoculated *Trichoderma* strain on the compost prepared according to the present work was high. We (re)isolated from the resulted compost fungi that have morphological characteristics and antagonistic properties similar to Td50b strain - Figure 4.

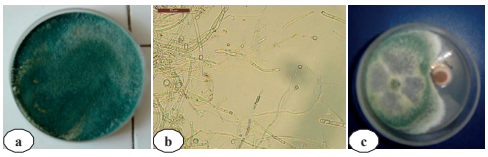

Figure 4. Different aspects of the re-isolated Trichoderma from Td50b inoculated compost: a) Morphological aspect of one isolate on PDA medium; b) Hyphae and spores on light microscopes; c) antagonism on PDA medium of *Trichoderma* isolate against *Fusarium graminearum* DSM 4527

The high rate of survival was promoted by the process that we developed. Our approach was to use a time released formulation of *Trichoderma* chlamydospores for the inoculation of the sawdust resulted from the extracted sea buckthorn branches. From such formulation *Trichoderma* propagules are continuous released, promoting the colonization of the substrate (Oancea et al., 2017b).

Production of volatiles regulates *Trichoderma* population by sporulation induction (Nemcovic et al., 2008) and maintains an additional reservoir of forms resistant to harsh conditions, able to recolonize the substrate in case of a drop of initial colonizing population.

The (re)isolated *Trichoderma* maintain its plant biostimulant characteristics, increasing the chlorophyll content of *A. thaliana* (cv. Columbia) - Table 2.

Table 2. The chlorophyll a and b content (mg per 100 g fresh weight) of *A. thaliana* seedlings (cv. Columbia) exposed for 48 h to the volatiles produced by the *Trichoderma* (re)isolate from compost inoculated with *T. harzianum* Td50b strain

	Chlorophyll a	Chlorophyll b
Control, not submitted to volatiles	12.47±1.38	3.24±0.37
Exposed for 48 h to the volatiles produced by *Trichoderma* (re)isolate from re-inoculated compost	18.62±1.92	3.94±0.28

Strain Td50b was demonstrated to enhance photosynthesis in the treated plant, by increasing chloroplast numbers and dimension and by higher contents of chlorophyll in the leaves (Şesan et al., 2020). Reisolated strains present similar effects on *A. thaliana* seedlings. Various type of composts were demonstrated to be suitable carriers for multipurpose *Trichoderma* strains. In the case of process, the Td50 strain, inoculated as alginate beads formulation, initially acts as compost accelerator, followed by a robust substrate colonization.

CONCLUSIONS

Treatment of the sea buckthorn branches sawdust, after the extraction of the polyphenols, with a time released alginate beads formulation of a multipurpose strain, *T. harzianum* Td50, accelerate the composting process. The resulted compost is a good substrate for further colonization. he proposed process recover the high-value ingredients from sea buckthorn branches and produce a compost that close the loop for mineral nutrients in sea buckthorn orchard in a sustainable manner, reduce the risk for proliferation of soil-borne pathogens producing sea buckthorn wilt/drying and increase the population of biostimulant *Trichoderma*.

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