Scientific Papers. Series A. Agronomy, Vol. LVI, 2013
ISSN 2285-5785; ISSN CD-ROM 2285-5793; ISSN Online 2285-5807; ISSN-L 2285-5785

MANAGEMENT OF BENEFICIAL MICROORGANISMS RESOURCES TO SUSTAINABLE AGRICULTURAL PRODUCTION

Liliana Aurora STEFAN, Alexandru PAICA, Cristian Iulian ENE

Research and Development Institute for Plant Protection, no. 8, Ion Ionescu de la Brad Blvd, District 1, 013813, Bucharest, Romania

Corresponding author email: aurora.stefan@yahoo.com

Abstract

The sustainability and security of Romanian agricultural production is under threat from intensive production practices, global competition and climate change factors. Maintaining the sustainable production of land lies in striking a balance between socio-economic goals and responsible natural resource management. To rise to the challenge, an integrated approach making full use of all the major players involved – farmers, consumers, regulatory bodies and scientists – is necessary.

Trichoderma pseudokoningii Td85 *antagonistic fungus was isolated from wheat seeds, Baragan agricultural region. Td85 beneficial strain has the following properties: a wide spectrum of action towards pathogenic microbiological contaminants in cereal crops, due to complex activity expressed by antagonism and competition for food and space colonization. Td85 beneficial strain provide reducing development of primary inoculum that survive in the soil and on the plant debris, due to high capacity for colonization and degradation of plant substrates.*

Saccharomyces cerevisiae L30b *yeast strain was isolated from grapes Chasselas D'ore variety, RDIPP Bucharest vineyard. Research of L30b beneficial yeast was sidelined on strawberry crop, focusing on biological control of Botrytis cinerea fungus. The efficacy of L30b formulations was assessed on Favette (early), Cardinal (middle) and Pandora (late ripening) variety, achieving a reduced disease incidence and severity of grey mould on fruit and providing high efficacy against the pathogen.*

Key words: *sustainable production, beneficial fungi, Trichoderma pseudokoningii Td85, Saccharomyces cerevisae L30b.*

INTRODUCTION

Microorganisms of the genus *Trichoderma* are some of the most common naturally occurring fungi. Most strains are beneficial and have ability to colonize plant roots easily, without damage them. Close relationship between plants and *Trichoderma* fungi, gives an excellent biocontrol agent qualities. These microbial biofungicide may compete for food and space with pathogenic fungi, may stimulate plant host defenses and increased positive effect for root system. In addition, antagonistic fungi have capacity to attack and parazitize agents of agricultural plant pest in certain environmental conditions (Sesan et al., 2012).

Fungi of the genus *Trichoderma* ssp. possess innate resistance to to most agricultural chemicals, including fungicides, although individual strains of microorganisms differ in terms of their resistance to plant protection products (PPPs). Some strains of antagonistic fungi were selected or modified to achieve specific agricultural chemical resistance. Most owners of strains of the genus *Trichoderma* ssp, hold extensive lists of their sensitivity or resistance to a wide range of crop protection products, for biological control agents of pest.

In nature, some organisms favors the development of others, forming associations that allow coevolution in the agriculture system, while others are exclude each other by mechanisms of antagonism. Microorganisms with importance in biological control of of plant diseases have a complex action.

Antagonism of biological control agents against pathogens is due to the action of secondary metabolites and to direct destruction by micoparazitism.

Reducing *Botrytis cinerea* pathogens may be due to the mechanism that destroy microsclerotium or limit mycelium growth by antagonistic yeasts of *Saccharomyces* and *Metschnikowia* genus.

MATERIALS AND METHODS

***Trichoderma pseudokoningii* Td85 strain,** beneficial microorganism, isolated from wheat seeds collected from Baraganul de Sud (Figure 1).

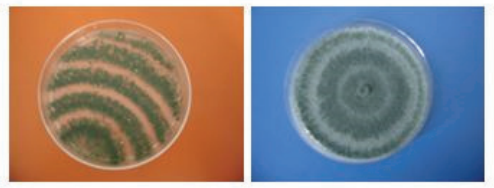

Figure 1. Pure cultures of beneficial fungus *Trichoderma pseudokoningii* Td85

Identification of *Trichoderma* Td85 strain to the species level was based on morphological and physiological characteristics, assigned to the species *Trichoderma pseudokoningii*.

Td85 colony habitat: colony on oatflake-agar at 25^0C filling the Petri-dish within 4 days, mycelium hialine, sporulating areas tuffed, green. Colony reverse colour unchanged. No odour. No growth on PDA at 40^0C; 50mm radius at 40^0C.

Td85 morphology: conidiophores tree-like, branched at right angles; length of branches increasing to the basis; no sterile appendages. Phialides flask shaped, straight or bent, arranged mostly in groups of three of the end of branches. Conidia elllipsoidal, smooth-walled, 5x3 µm.

Lyophilized *Trichoderma pseudokoningii* Td85 strain Figure 2).

Figure 2. Vials of Td85 strain as lyophilized spores

Condition for storage of beneficial microorganisms Td85 are lyophilization (freeze-drying cell) and for cultivation PDA medium.

***Saccharomyces cerevisiae* L30b strain**, beneficial microorganism, isolated from *Chasselas D'ore* grapes variety, RDIPP vineyard (Figure 3, 4 and 5).

Figure 3. Habitat of L30b strain and yeast collony isolated from Chasselas d'ore grapes variety

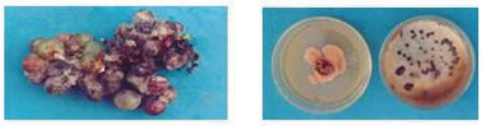

Figure 4. Cultures obtained on PDA medium of Saccharomyces cerevisie L30b and *Botrytis cinerea*-the target pathogen in strawberry experimental field

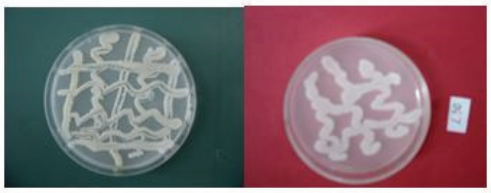

Figure 5. Pure cultures of beneficial yeast *Saccharomyces cerevisiae* L30b strain

The culture medium used for isolation: glucose 5%, 2% agar, 0.5% tartaric acid and pH 2.5 and for cultivation: YPGA medium (glucose 20g, peptone 10g, 5g yeast extract, 20g agar; sterile distilled water (1000 ml).

The criterion used for the proposed taxonomic designation for L30b yeast strain is the key to the species of *Saccharomyces* adapted from Stelling-Dekker.

Morphology of *Saccharomyces* L30b strain *surface on agar*: shiny; *texture on agar:* smooth.

Identification of L30b yeast at the species *Saccharomyces cerevisiae* was performed by sequence analysis of 26S rDNA D1/D2. Identification species: *Saccharomyces cerevisiae* (100% sequence D1/D2).

Conditions for storage are lyophilization and cryopreservation (storage in liquid nitrogen or mechanical freezing at temperatures between-80^0C and-135^0C).

Lyophilized *Saccharomyces cerevisiae* L30b strain stored into international collection of

microorganisms DSMZ Braunschweig – Germany, Figure 6).
Mineralization capacity of Td85 strain on plant debris was assessed by Strathox respirometer.
Pest incidence (% infected fruit) and pest severity (% infected area) of *Botrytis cinerea* was assessed on 100 fruit per plot. The results were interpreted statistically by ANOVA and Newman & Keuls 5% test.

Figure 6. Vials of L30b yeast strain as lyophilized spores

RESULTS AND DISCUSSIONS

Experiments conducted in this research allowed the definition of optimized conditions (composition of media, cultivation parameters-temperature, aeration) and conditioning formula that provides optimum performance in growing and multiplication replicates antagonistic strains to phytopathogenic microorganisms
Modern crop protection products must fulfill the requirements increasingly higher efficiency requirements in the field, but with minimal impact on the environment and the highest possible level of safety for both, the consumer and food products. Eliminate risks to health and environment of plant protection products is a major imperative for any company producing PPPs. Product behavior in soil, water and air and their effects on the fauna and flora are thorough tested in greenhouses and in the field, following international regulations for sustainable development.

BIOPRODUCTS BASED ON *Trichoderma Pseudokoningii* Td85 TO PROTECT CROPS

Different conditioning formulas and modes of actions: controlled release granules (F1 left) microgranules with curative action (F2 midl.) powder conditioning of seed treated with preventive actions (F3 left) (Figure 7). Active ingredient: *Trichoderma pseudokoningii* Td85 strain ($1x10^8$ spores/ml).

Figure 7. Bioproducts based on *Trichoderma pseudokoningii* Td85 strain with different conditioning formulas

Microgranules viability of Td85 spores was 100% after 12 months of conditioning and more than 90% viable spores, demonstrating the high level of conditioning formulas in yield (Figure 8 and 9).

Figure 8. Viable conidia of Td85 strain after 4 (left) and 8 (right) days from conditioning as controlled release granules (F1)

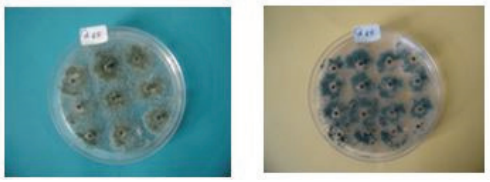

Figure 9. Viability of bioproducts based on Td85 strain embedded in alginic acid (F1) and sodium alginate (F2) after 12 month from conditioning

For testing the viability, embedded Td85 spores are transplanting on PDA medium and growned for 5 days at 22^0C. Alternatively, a pure fungal suspension from a tube is tested to quantify microscopic spore viability (hemacytometer+methylviolet/methylene blue), considering spores viable, if more than 90% of the spores is not colored.
Biological activity of strain Td85 is differentiated according to the formula of beneficial microencapsulation. (Figure 10 and 11). Our research publicated in Patent application a 2010 01161 demonstrated that controlled slowly releasing of Td85 spores (F1) are applicable in a conservative agriculture system.

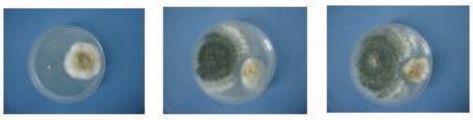

Figure 10. Controlled slowly releasing of Td85 spores and the antagonism against *Fusarium graminearum*/ Td85 F1 (a) Td85 F2 (b) and control fungus (c)

Figure 11. Instant releasing spores and the antagonism of Td85 against *Fusarium graminearum* / Td85 F1 (a) F2 Td85 (b) control fungus (c)

The selection criteria of beneficial microorganism *Trichoderma pseudokoningii* Td85 are: antagonism towards different agents of damages; ensure reducing primary inoculum developed on plant debris from agricultural crops; high capacity for colonization and degradation of plant substrates; a complex action expressed by: antagonism, competition for food and space, high capacity of sporulation, submerged cultivation, in order to obtain biomass because it is an industrial profitable technique readily taken up.

To define optimal industrial parameters of *Trichoderma pseudokoningii* Td85 strain, the following composition was used: KH_2PO_4 5 g/l, $(NH_4)_2SO_4$ 5 g/l, yeast extract 2 g/l, $MgSO_4.7H_2O$ 0.3 g/l, $CaCl_2.2H_2O$ 0.3 g/l, whey powder 5 g/l, pulp corn to produce bioethanol by dry milling about 5 g/l, pH 5.6.

The results based on our research publicated in Patent application a 2010 01161 and 127293 demonstrated that *Trichoderma pseudokoningii* Td85 has antagonistic action against pathogens from *Fusarium, Botrytis* and *Sclerotinia* genus. Also, Td85 strain is producing hydrolases that degrades cellulose, chitinase and beta-glucanase; oxidase which degrade lignin and has high mineralization capacity of plant debris (Figure 12).

Figure 12. Determining of mineralizarion capacity of Td85 strain on wheat debris

BIOPRODUCTS BASED ON YEAST *Saccharomyces cerevisiae*L30b TO PROTECT CROPS:

Different conditioning formulas and modes of actions: soluble granulas into sodium alginate (F1); soluble granulas into sodium bicarbonate (F2); mixed bioproduct based on *Saccharomyces* and *Metschnikowia* yeasts (Figure 13).

Figure 13. Bioproducts based *on Saccharomyces cerevisiae* L30b strain with different conditioning formulas

To obtained biomass of L30b yeast, the following biosinthesys composition was used (all amounts are per litre) : 30 g of plasmolizate syrup, 5g yeast plasmolizates (supplemented with iron), 2g of $(NH_4)_2HPO_4$, 1g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$ (Figure 14).

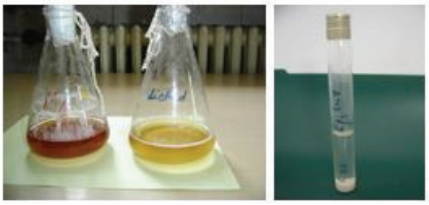

Figure 14. Biosynthesis of L30b yeast biomass used as cell suspension at different dilution rates

For the industrial parameters, plasmolizate syrup and yeast were determined following the optimum growing of L30b strain: 33°C temperature and an aeration rate of 0.75 l air/l medium/min (Oancea F. et. all., Patent 01382 and 01383, 2012).

Design, analysis and reporting of the strawberry trials in accordance with the European and Mediterranean Plant Protection Organization (*EPPO*) *guidelines 152 and 181,* regarding *q*uality assurance program for agricultural testing in the field, in greenhouses and in laboratories condition.

Protocol of research and reporting in accordance with Good Experimental Practice and Directive 93/71/EEC.

by preventive treatments included: tested Efficacy study of *Saccharomyces cerevisiae* L30b yeast against *Botrytis cinerea* (grey mold) bioproducts Saccharopulvin 25 PU with yeast cell at different dilution rates ($2x10^6$ and $6x10^6$ cell/ml); reference products (thiophanate methyl 0.07%) and an untreated control.

The efficacy of L30b formulation was assessed on Favette (early), Cardinal (middle) and *Pandora* (late ripening) variety on areas of 100 m^2/plot with 3 replicates.

Figure 15. Biological experimental in strawberry field; Favette (early), Cardinal (middle) and Pandora (late ripening) varieties

During strawberry vegetation period, 2 treatments were applied in the following phenophases:

T_1: After flowering fruit-binding (when there are a number of physical and chemical processes that lead to the creation of a favorable environment for the development B. cinerea fungus.

T_2: with 2-3 weeks before harvest to protect the fruit from lately attack of *B. cinerea*.

Observations were made on strawberry fruit before harvest.

L30b strain used as biological control agent of preharvest strawberry diseases has protection activity against *Botrytis cinerea* pathogen, the causal agent of gray mould (Figure 16).

Saccharomyces cerevisiae L30b yeast was effective against gray mold (*Botrytis cinerea* during strawberry vegetation period, applied as Saccharopulvin 25 PU bioproduct with cell suspension at different dilution rates ($2x10^6$ cell/ ml and $6x10^6$ cell/ ml).

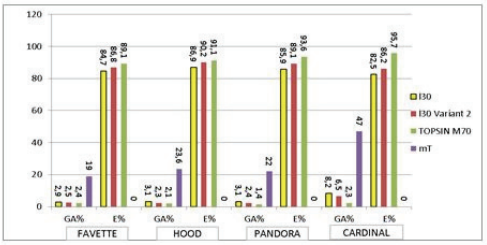

Figure 16. Efficacy of Saccharopulvin 25 PU bioproduct against Botrytis cinerea in strawberry experimental field

Bioproduct Saccharopulvin 25 PU ($6x10^6$ spores/ml), in field conditions, recorded highest efficacy (E = 86.8 to 90.2%) compared to the efficacy of chemical standard thiophanate methyl (E = 89.1 to 95.7%) for Favette, respectively, Pandora variety (ANOVA and Newman & Keuls 5% test).

The attack level during efficacy trial, ranged between 19.0 to 47.0% for Favette respectively, Pandora variety.

Bioproduct Saccharopulvin 25 PU maintain strawberry fruit quality properties and does not affect the organoleptic properties when applied two treatments in vegetation (Based on our research published in patent no. 125071).

The economic implications of bioproducts: an alternative to chemical treatments avoiding the formation of breeds resistant to fungicides, compatibility with integrated control systems, reducing quantitative and qualitative losses through high efficacy, not necessary breaks.

CONCLUSIONS

Trichoderma pseudokoningii Td85 strain is a solution applicable in a conservative agriculture system.

Td85 strain has high mineralization capacity of plant debris based on our reasearch published in patent no. 127293 and antagonistic properties against soil-borne pathogens, reducing the primary inoculum level of microbiological pest agents.

Saccharomyces cerevisiae strain has biological activity against *Botrytis cinerea* pathogen, achieving a reduced disease incidence and severity of grey mould on strawberry and providing high efficacy against the fungus.

The both beneficial microorganisms in agricultural crops, owned by RDIPP Bucharest are stored into International collection of

microorganisms *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ) in Braunschweig, Germany.

Trichoderma pseudokoningii Td85 strain DSMZ access number 23661/2010, *Saccharomyces cerevisiae* L30b strain DSMZ access number 23648/2010.

ACKNOWLEDGEMENTS

This research work was carried out with the support of: Dr. Oancea Florin, project manager of MAKIS and BIOTECH 4630; Agricultural Research and Development Station Caracal, experimental field of Td85 strain; Research and Development Station for Fruit Tree Growing Baneasa, Bucharest, experimental field of L30b yeast.

REFERENCES

Oancea F., Stefan A.L., 2011. Yeast strain L30 *Saccahromyces cerevisiae* antagonistic to microbiological pest. Patent RO 125 071 / 28.10.2011.

Oancea F., Stefan A.L., Lupu C., 2010. *Trichoderma pseudokoningii* strain for systems of conservative agriculture. Patent application A/2010/01161/ 24.11.2010.

Oancea F., Stefan A.L., Lupu C., 2013. *Process of growing plants in mulch bioactive*. Patent application A 2013 127293 / 29.03.2013.

Oancea F., Stefan A.L., Lupu C., 2012. Antagonistic yeast encapsulation process, drying and conditioning process of encapsulated yeasts recovered after alcoholic fermentation and bioproduct resulted from their use. Abstract published in the Official Bulletin of Industrial Property-Patents Section No. 6 / 29.06.2012. Patent application a 2010 01383 / 21.12.2010.

Oancea F., Stefan A.L., Lupu C., 2012. Yeast strain with antagonistic and fermentation activity. Abstract published in the Official Bulletin of Industrial Property-Patents Section No. 6 / 29.06.2012. Patent application a 2010 01382 / 21.12.2010.

Sesan T.E., Oancea F., Stefan A.L., Lupu C., Iliescu H., 2012. Antagonistic strain of *Trichoderma viride* and process to obtain of antifungal bioproduct based on it. Patent no. 126125/28.12.2012 OSIM.