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SURFACE RESPONSE OPTIMIZATION OF SUBMERGED BIOMASS PRODUCTION FOR A PLANT BIOSTIMULANT Trichoderma STRAIN

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Abstract

We describe the optimization of cultivation medium composition for biosynthesis of Trichoderma asperellum T36 biomass. This Trichoderma strain is a multifaceted one, being antagonist against major plant pathogens, stimulating vegetables growth, enhancing bioactive accumulation into nutraceutical crops and promoting development on early stages of the plants cultivated into high residues system. To improve the eco-efficiency of T36 based bioproducts production, a maximum conversion of cultivation medium components into fungal biostimulant biomass is required. We optimize the cultivation medium through a designed experiment, based on surface response methodology, wherein several components of cultivation medium were modified in the same time. The studied components were: glucose (carbon source), ammonium sulphate (inorganic nitrogen and sulphur source), soymeal and yeast extract (complex, organic nitrogen source and growth factors source), potassium mono- and di-hydrogen-phosphates (as phosphorus source and buffering ingredients). The optimal composition of cultivation medium, resulted after experimental results analysis, is: 34.2 g. l^{-1} glucose, 0.37 g. l^{-1} ammonium sulphate, 0.8 g. l^{-1} yeast extract, 2.7 g. l^{-1} soymeal, 1.2 g. l^{-1} K_2HPO_4, 1.7 g. l^{-1} KH_2PO_4. We compared the tolerance to dry-flowable formulation of T. asperellum T36 biomass produced on optimized liquid media and on liquid Weidling media. The biomass produced on optimized media has a better tolerance to dry-flowable formulation than biomass produced on Weidling media. Also, the preservation of biological activities, specific to T36 strain, is better on dry-flowable formulation produced with biomass resulted from optimized medium than that with biomass resulted from liquid Weidling medium. Higher tolerance to dry-flowable formulation and better survival rate, of formulated T36 propagules, suggest that optimized medium promote formation of more resilient fungal resting structures. A techno-economic analysis was performed for the optimized cultivation medium.

Key words*: Trichoderma, biostimulant strain, cultivation medium, optimization, surface response methodology.*

INTRODUCTION

Despite positive effects associated with the use of agrochemicals on costs and yield efficiency in various agriculture systems, it is now recognized that this practice presents major environmental sustainability issues (Carvalho, 2006; Gomiero *et al.*, 2011).

Among alternatives to agrochemicals, with significant lower potential impact on environment, are included microbial based bioproducts (Pérez-García *et al.*, 2011).

Strains from *Hypocrea* genera, usually known by the name of their anamorph forms, *Trichoderma*, are the most used fungal active ingredients into such agricultural microbial products, being marketed as biopesticides, biofertilizers, plant strengtheners and plant biostimulants (Woo *et al.*, 2014).

Initially *Trichoderma* strains, selected on their *in vitro* antagonism against fungal plant pathogens, were proposed to be used as biopesticides/biofungicides, due to their proven high biocontrol efficacy on field trials (Sesan *et al.*, 1999; Elad, 2000; Benitez *et al.*, 2004). However, the plant protection effects of fungal strains from *Trichoderma* genera are not related only to biocontrol through microbial antagonism. *Trichoderma* was considered also an opportunistic avirulent plant symbiont, which activate plant defence mechanisms (Harman *et al.*, 2004). Stimulation of natural

mechanisms involves both jasmonic acid-ethylene (JA/ET) and salicylic acid (SA) signalling pathways (Nawrocka and Malolepsza, 2013) and is done through the action of various elicitors, including MAMPs, microbial-associated molecular patterns, and DAMPs, damage-associated molecular patterns, (Hermosa et al., 2013).

Several *Trichoderma* strains have been marketed as biofertilisers (Kaewchai *et al.*, 2009), mainly due to their effects on phosphate and micronutrients bioavailability (Altomare *et al.*, 1999) or on nutrients (mainly nitrogen) use efficiency (Harman, 2011). However, plant growth promotion by *Trichoderma* strains results also from other mechanisms, beside enhanced nutrient availability / use efficiency. Such mechanisms include production of plant hormones, as indole acetic acid, indole-3-carboaldehyde, indole-3-acetaldehyde, indole-3-ethanol (Contreras-Cornejo *et al.*, 2009), plant hormones modulators like ACC-deaminase (Zhang *et al.*, 2017) or bioactive secondary metabolites – e.g. harzianolide and 6*n*-pentyl-6*H*-pyran-2-one (6PP) (Vinale *et al.*, 2008), chrysophanol (Liu *et al.*, 2016), cremonolide (Vinale *et al.*, 2016).

The above mentioned multilevel actions on plants of plant beneficial fungal *Hypocrea* / *Trichoderma* strains was proposed to be better described as plant biostimulants activities (Lopez-Bucio *et al.*, 2015). Plant bio-stimulants represent a new category of agricultural inputs, which enhance plant tolerance to biotic and abiotic stress, promote plants growth, improve nutrient uptake and nutrient use efficiency, influence yield quality (Brown and Saa, 2015; du Jardin, 2015). Despite their regulatory status, which is not fully defined on important markets, e.g. European Union, plant biostimulant market will reach $2,241million by 2018, with a compound annual growth rate of 12.5% (Calvo *et al.*, 2014).

Our group focused on the last years on selection and applications of *Hypocrea* / *Trichoderma* plant biostimulants strains (Oancea *et al.*, 2014; Răut *et al.*, 2015; Şesan *et al.*, 2015). We developed new formulations for our selected plant biostimulants *Trichoderma* strains, a dry-flowable formulation (Oancea *et al.*, 2016a) and a hydro-gelified and film forming formulation (Oancea *et al.*, 2016b), intended to be used mainly on conservation agriculture/high residues farming and for nutraceutical crops.

The holistic approach of sustainability for agro-systems (Arodudu *et al.*, 2017) involves the eco-design of used inputs, aiming an increased eco-efficiency, with a lower carbon footprint. For manufacturing process of bioproducts, especially for those based on *Trichoderma* biostimulant strains and intended to be used into low inputs agricultural systems, eco-efficiency requires also a maximum conversion of low costs cultivation media ingredients into fungal biomass / fungal propagules.

The main objective of this study was to optimize, in term of submerged fungal biomass / fungal propagules productivity, the composition of a liquid cultivation media, for a *Trichoderma* biostimulant strain from our collection, *T. asperellum* T36, through a designed experiment, based on surface response methodology. A first derived objective was to establish the influence of submerged cultivation, into resulted medium with optimal composition, on T36 propagules tolerance to formulation, T36 propagules survival during storage of formulated bioproduct and preservation of T36 specific activities during bioproduct storage. Final derived objective was to make a techno-economic analysis of the biostimulant *Trichoderma* biomass production, on the optimized medium.

MATERIALS AND METHODS

Plant biostimulant Trichoderma strain. We used on our study a strain from ICECHIM collection, *T. asperellum* T36 NCAIM F 001434, a multifaceted, plant biostimulant strain. This strain is antagonist for major plant pathogens (Răut *et al.*, 2014b), produces bioactives volatile compounds, including 6PP (Răut *et al.*, 2014a), protects and stimulates vegetables growth (Răut *et al.*, 2016), accelerate the degradation of lignocellulose material, enhances bioactive accumulation into nutraceutical crops and promotes development on early stages of the plants cultivated into high residues system (Răut *et al.*, 2015).

Optimization of cultivation medium composition. We optimize the cultivation

medium through a designed experiment, based on surface response methodology, wherein several components of cultivation medium were modified in the same time. These studied components were: glucose (carbon source), ammonium sulphate (inorganic nitrogen and sulphur source), soymeal and yeast extract (complex organic nitrogen and growth factors source), potassium mono and di-hydrogen-phosphates (as phosphorus source and buffering ingredients). Design of Experiments (DoE) approach was utilized for optimization of biomass production. Cultivation medium components, considered as independent factors into DoE, were coded as following: glucose-X1; ammonium sulphate-X2; dipotassium phosphate-X3; mono-potassium phosphate-X4; yeast extract-X5; soymeal-X6, were assigned as independent variables, as shown in Table 1. Each independent factor was tested at three levels, with -1 designated as the lower level, 0 as the centre and +1 as the upper level. Twenty individual experiments were performed with different factor configurations. We used a specialised software, Design Expert® v.10.0 (Stat-Ease, Minneapolis, MN, USA), as support for experiments design.

Table 1. Independent factors considered for the biomass optimization of *T. asperellum*

Factor (g/l)	Name	Factorial levels		
		–1	0	1
X1	glucose	15	25	35
X2	$(NH_4)_2SO_4$	0.20	0.30	0.40
X3	K_2HPO_4	1	1.2	1.4
X4	KH_2PO_4	1	1.4	1.8
X5	yeast extract	0.5	0.7	0.9
X6	soymeal	1	2	3

To ensure reliable data collection and evaluation, several non-variable factors were taken in consideration. The *T. asperellum* T36 strain was cultivated in a submerged medium under continuous aeration and stirring. On each experiment 100 ml of liquid medium was distributed in 500 ml Erlenmeyer flasks, sealed with cotton plugs. The flasks were incubated at 25°C and agitated at 100 rpm. After 7 days, the resulted biomass was separated from the culture medium using an ultra-filtration membrane. The resulted biomass was weighted after drying at 105°C for 4 hours.

Dry-flowable formulation. We used a process for dry-flowable formulation, described in details elsewhere (Oancea *et al.*, 2016a). Briefly, this process includes the following main steps: (*i*) encapsulation of biostimulants *Trichoderma* biomass into soft alginate microbeads; (*ii*) drying encapsulated fungi with antioxidant protection and (*iii*) mixing the resulted spray-dried flowable powder, with polyvinyl alcohol, as adhesive agent, lecithin as wetting agent and a CO_2 generating dispersant system, based on polyacrylic acid, citric acid and sodium bicarbonate. We applied this dry-flowable formulation process to T36 wet biomass, obtained after cultivation for 7 days, at 25°C and 100 rpm, from resulted DoE optimal media or from liquid Weidling medium, as was already presented (Sesan and Oancea, 2010).

Assay of water activity on stored dry-flowable formulation. We determined the water activity on the dry-flowable formulations, made with T36 biomass produced on resulted DoE optimal medium and on liquid Weidling medium, by using a water activity meter (4TE, AquaLab, Pullman, WA, USA). These determinations were done each two month, for bioproducts maintained on room temperature, on closed dark plastic bottles.

Determination of viable propagules. We performed monthly determination of viable propagules, on the stored dry-flowable formulations, made with T36 biomass produced on DoE optimal medium and on liquid Weidling medium. The determination was done by cultivation on a selective media (Williams *et al.*, 2003), which contains, beside the minimal nutrients sources, biocides with selective action toward *Trichoderma* strains: 1.5 $ml.l^{-1}$ formulated propamocarb (Previcur 607 SL, Bayer Crop Science, 607 g active ingredient per litre); 0.15 $g.L^{-1}$ Roz Bengal (sodium salt, R3877 Sigma, Sigma–Aldrich), 0.2 $g.L^{-1}$ pentachloronitrobenzen (quintozene, P2205 Sigma, Sigma-Aldrich), 9 $ml.L^{-1}$ of stock solution of streptomycin (1% weight/volume, S6501 Sigma-Aldrich), 0.25 g chloramphenicol (C0378 Sigma-Aldrich). For these determinations, we used, beside the reagents supplied by mentioned producers, reagents from EMD Millipore.

Determination of in vitro antagonistic characteristics. We determine the antagonistic activity, resulted from volatiles produced by dry-flowable formulations based on T36, grown on potato-dextrose agar, by using the double plate sandwich confrontation assay (Răut *et al.*, 2014a). Into this confrontation assay the used plant pathogen fungal strain was *Fusarium graminearum* DSM 4527. We made these determinations monthly, on the room temperature stored T36 formulations, obtained with biomass produced on DoE optimal medium or on liquid Weidling medium.

Assay of 6PP production. We sampled 0.1 g of each dry-flowable formulations, immediately after formulation process and after 12 month of storage. The formulation samples were cultivated on potato-dextrose broth for 10 days. We homogenized with a blender (Waring®, Laboratory Blender, Fischer Scientific, Waltham, MA, USA) the resulting mycelium within culture medium. We determined the biomass from homogenate gravimetrically, after filtration on filter paper Whatman No. 1 and drying at 105°C. The homogenate was extracted twice with dichloromethane, $CHCl_2$, 1 part of homogenate to 1 part of $CHCl_2$. We reunited the extracts, we vacuum dried (Rotavapor, Buchi, Flawil, Switzerland) and we re-suspended in 0.1 ml $CHCl_2$. We determined the 6PP content by gas-chromatography (El-Hasan *et al.*, 2007), using an Agilent 700 gas chromatograph, equipped with quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) and a standard curve, made with pure 6PP (Sigma-Aldrich).

Preservation of plant material degradation activity. We organized an experiment wherein we recorded the oxygen consumption and release of various compounds from lignocellulose material, treated with the tested dry-flowable formulations, made with biomass produced on DoE optimal medium or on liquid Weidling medium. We introduced aseptically into sterile Erlenmeyer flasks the following: 0.1 g of lignocellulose material (wheat stem, dried, grounded to 0.25 mm and sterilized by gamma-irradiation), 19 ml of sterile water, pH 5.5, and 0.1 g of flowable formulation, with $5x10^9$ cfu per g. We determined the oxygen consumption after incubation for 48 hours on room temperature, by using an oxygen fluorescence probe (Ocean Optics, Halma, Amersham, UK). We recovered the supernatants and we determined: reducing carbohydrates, with DNS reagent (King et al., 2009); Total Organic Carbon (TOC), with a HT Formacs apparatus (Scalar Analytical, Breda, Netherlands), using the batch method (Trulleyová and Rulík, 2004) and total soluble phosphorus (Self-Davis and Moore Jr, 2000). We performed the experiments with a control, which include same determinations, done on the lignocellulose material not-inoculated with dry-flowable formulations based on *Trichoderma* plant biostimulant strains. We made these determinations on the beginning and on the end of 12 months' storage period.

Techno-economic analysis. We used a model for *Trichoderma* industrial cultivation previously developed for cellulases pro-duction (Klein-Marcuschamer *et al.*, 2012), based on a cascade of batch biosynthesis, each bioreactor providing 5% inoculum to the next bioreactor in the cascade. Stoichiometry of the conversion of culture medium components into plant biostimulant *T. asperellum* T36 biomass is that calculated per bellow Equation 1. The residence time for each bioreactor was 96 h. We considered the costs of the main agro-industrial raw materials, glucose, soymeal and yeast extract, used for the *Trichoderma* growing media, as being the average price for such agricultural commodities on European Union during the last year (EC, 2017). For inorganic ingredients of cultivation medium, ammonium sulphate and potassium mono- and di-hydrogen-phosphates, we considered the price for fertilizers (EC, 2017). Costs were expressed in USD.

Statistical analysis. We made all experiments in triplicate, in complete randomized blocks. Colony forming units, cfu, per g or per ml, were log-transformed, with the calculation of standard errors. We used comparison on average survival percentage and not in log cfu. g^{-1}, because propagules producing biomass was produced from different batches. We established statistical relevance by ANOVA and linear mixed model (Bolker *et al.*, 2009). We used a specialised software, Design Expert® v.10.0 (Stat-Ease, Minneapolis, MN, USA), for analysis of the results from designed experiments based on surface response

methodology. We used Excel software (Office 365 - Excel 2016, Microsoft, Redmont, WA, USA) to make calculations and to draw figure.

RESULTS AND DISCUSSIONS

We performed twenty individual experiments with different factor configurations, based on independent factors, codes as in Table 1, which produced different quantities of biomass, expressed as dry biomass, $g.l^{-1}$ (Table 2). Even on the most effective independent factor combination *Trichoderma* dry biomass yield is still under threshold of 10 $g.l^{-1}$. We analysed the experimental data containing the independent factors, by using a specialised software, Design Expert® v.10.0 (Stat-Ease).

Table 2. Independent factors and corresponding three level design space

Test	Independent variables						Biomass
	X1	X2	X3	X4	X5	X6	($g.l^{-1}$)
1	–1	–1	–1	–1	–1	–1	6
2	1	–1	–1	–1	1	–1	6,1
3	–1	1	–1	–1	1	1	7,8
4	1	1	–1	–1	–1	1	8,4
5	–1	–1	1	–1	1	1	7,2
6	1	–1	1	–1	–1	1	7,4
7	–1	1	1	–1	–1	–1	9
8	1	1	1	–1	1	–1	7,9
9	–1	–1	–1	1	–1	1	6,5
10	1	–1	–1	1	1	1	6,9
11	–1	1	–1	1	1	–1	8,6
12	1	1	–1	1	–1	–1	7,5
13	–1	–1	1	1	1	–1	7,8
14	1	–1	1	1	–1	–1	6,3
15	–1	1	1	1	–1	1	9
16	1	1	1	1	1	1	9,1
17	0	0	0	0	0	0	7,3
18	0	0	0	0	0	0	7,1
19	0	0	0	0	0	0	7,2
20	0	0	0	0	0	0	7,2

We established a full factorial DoE, with two levels. We arrive to a reduced 3FI design model, by imposing a constraint of 20 levels, which include 4 centre points. Model effects were determined from the half-normal plot. These include: X1, X2, X3, X4, X6, X1:X3, X1:X4, X1:X6. The last three terms show that the glucose content in the beginning interacts statistically with the K_2HPO_4, KH_2PO_4 and soymeal levels. We can also observe that the yeast extract content (X5) does not interact significantly with the glucose content.

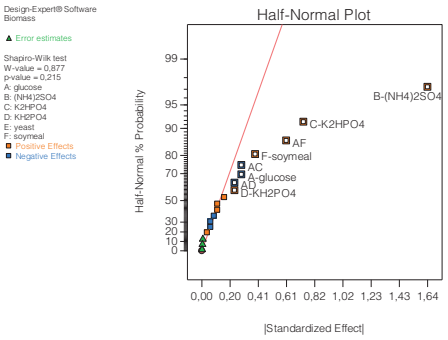

Figure 1. Half-normal plot generated by Design Expert 10 for determining individual term effects

We made an ANOVA analysis, to assess how well the proposed statistical model fits. In Table 3 we can see the calculated p-value for the model and for each individual term.

Table 3. P-values for model and individual terms

Source	Sum of Squares	df	Square	Value	p-value
Model	16.12	8	2.01	28.03	< 0.0001
X1	0.33	1	0.33	4.6	0.0551
X2	10.73	1	10.73	149.2	< 0.0001
X3	2.18	1	2.18	30.27	0.0002
X4	0.23	1	0.23	3.14	0.1041
X5	0.6	1	0.6	8.36	0.0147
X1*X3	0.33	1	0.33	4.6	0.0551
X1*X4	0.23	1	0.23	3.14	0.1041
X1*X6	1.5	1	1.5	20.88	0.0008
Residual	0.79	11	0.072		
Lack of Fit	0.77	8	0.096	14.45	0.0252
Pure Error	0.02	3	6.67E-03		
Cor Total	16.91	19			

The values are considered acceptable and validate the data resulted for the optimal composition of cultivation medium used for biosynthesis of T. *asperellum* T36 biomass. The coefficient of determination (R^2) is high enough (0.9532) for using the model for optimization purposes. The standard deviation is 0.27, while the coefficient of variation is 3.57, which further validates the model.
The final equation regarding actual factors was calculated and is presented here as Eq. 1.

$$Biomass = 4.558 - 0.013X1 + 0.062X2 + 0.08X3 + 0.238X4 - 0.242X6 - 1.438E - 003 \cdot X1 \cdot X3 - 5.938E - 003 \cdot X1 \cdot X4 + 0.01 \cdot X1 \cdot X6$$

Eq. 1

The optimal composition of cultivation medium, resulted after experimental data analysis with Design-Expert® v10.0, is: 34.2 g. l^{-1} glucose, 0.37 g. l^{-1} ammonium sulphate, 0.8

g. l^{-1} yeast extract, 2.7 g. l^{-1} soymeal, 1.2 g. l^{-1} K_2HPO_4, 1.7 g. l^{-1} KH_2PO_4. This composition is closed to the upper level of the considered independent factors and is on agreement with others recently reported optimization of biosynthesis processes based on *Trichoderma* strains, wherein low costs meals, from oleaginous seeds, were used as complex nitrogen sources of cultivation media (Gao *et al.*, 2013; Almeida *et al.*, 2015).

We further used the optimal medium composition, resulted from designed experiments based on surface response methodology (DoE optimal medium), for dry-flowable formulation.

The formulation process was done by using an already published process (Oancea et al., 2016a), which includes the following main steps: (*i*) encapsulation of biostimulants *Trichoderma* biomass; (*ii*) drying encapsulated biomass; and (*iii*) mixing the resulted spray-dried flowable powder with formulation ingredients. We applied this dry-flowable formulation process on T36 wet biomass, obtained on DoE optimal medium, after cultivation for 7 days, at 25°C and 100 rpm, or on liquid Weidling medium, as was already described (Sesan and Oancea, 2010).

We determined on stored dry-flowable bioproducts, obtained with T36 biomass produced on DoE optimal medium or liquid Weidling medium, the water activity, the number of propagules and the antagonistic activity resulted from the production of volatiles compounds. A slightly increasing on water activity, which remains well below the threshold requested for xerophyte micro-organisms (i.e. osmophilia yeasts) development into solid substrates, was demonstrated for both bioproducts stored for 12 months - Figure 2. Both bioproducts are stable and without risk to develop spoilage microorganisms.

Low water activity in our dry-flowable formulations is in concordance with others reports regarding formulation of spray-dried *Trichoderma* biomass (Jin and Custis, 2011; dos Santos *et al.*, 2015).

The survival of propagules from *T. asperellum* T36 biostimulant strain was good, for the tested period of storage of 12 months, on both bioproducts. During this storage period the T36 strain formulated into bioproducts maintained its antagonistic activity toward *F. graminearum*, resulted from the production of volatiles compounds active against fungal pathogens (Figure 3).

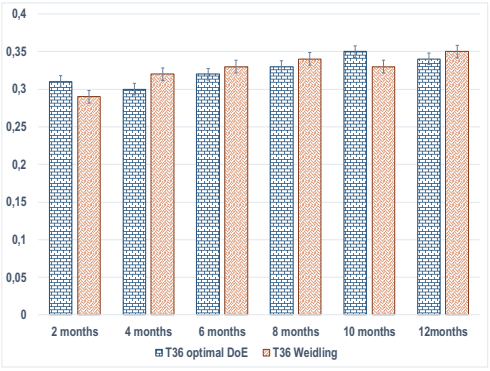

Figure 2. Evolution of the water activity of bioproducts, dry-flowable formulations of *T. asperellum* T36 biomass produced on optimal DoE medium or on Weidling medium

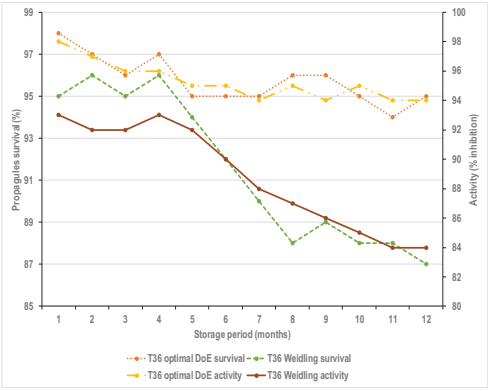

Figure 3. Survival and biological activity (% inhibition of growth of *F. graminearum* DSM 4527 by volatile) during storage of T36 propagules, produced on DoE optimal medium or on liquid Weidling medium and formulated as dry-flowable products

The survival rate and the biological activity against *F. graminearum* was significantly better for bioproduct which was made with biomass from DoE optimal medium. We determined also the preservation of the capacity to produce one of the major bioactive volatile compound, 6PP, 6*n*-pentyl-6*H*-pyran-2-one, which is toxic for fungal pathogens (El-Hasan *et al.*, 2007) and have a stimulant effect on plants (Vinale *et al.*, 2008). Both tested bioproducts retained their ability to produce 6PP after storage for 12 months – Table 4. *Trichoderma* propagules resulted from DoE

optimal medium preserved better the capacity to produce 6PP after storage as formulated bioproduct. The data suggest that plant-based, complex nitrogen sources, with high content of other bioactives ingredients, are more effective in promoting accumulation of fungal resting forms.

Table 4. Production of 6*n*-pentyl-6*H*-pyran-2-one (mg. g^{-1} dry mycelia) by T36 strain recovered from bioproducts made with propagules produced on DoE optimal medium or on liquid Weidling medium

T. asperellum T36 DoE optimal		*T. asperellum* T36 Weidling	
Initial	After 12 months	Initial	After 12 months
84.38±14.65	74.12±9.27	82.41±12.38	54.64±10.78

Production of 6PP by our *T. asperellum* T36 biostimulant strain, recovered from dry-flowable bioproducts, is on the same level with those already reported for other *Trichoderma* strains (Kalyani *et al.*, 2000; Serrano-Carreón *et al.*, 2004; El-Hasan *et al.*, 2007). Cultivation on DoE optimal medium promote formation of propagules which maintain better the ability to produce 6PP after long term storage on dry-flowable formulation – Table 4.

Our plant biostimulants T36 strain was intended to be used for treatment of plant residues which are covering the soil on conservation agriculture systems (Răut *et al.*, 2015), thus we tested also the preservation of the capacity of propagules, produced on DoE optimal medium or on liquid Weidling medium, to degrade lignocellulose material. The obtained results demonstrate that DoE optimal medium produce propagules which are preserving better the ability to degrade lignocellulose material specific to CA system, i.e. wheat straw – Table 5.

Table 5. Capacity of *Trichoderma* biostimulants strains, formulated as dry-flowable products, to degrade lignocellulose material

Determination	*T. asperellum T36* DoE optimal		*T. asperellum T36 Weidling*	
	Initial	After 12 months	Initial	After 12 months
Oxygen consumption ($mg.dm^{-3}$)	2.29±0.22	2.18±0.27	2.24±0.28	1.52±0.24
Soluble phosphorus ($mcg.dm^{-3}$)	18.76±2.12	17.84±1.54	18.58±1.74	14.92±1.81
Total organic content on supernatant ($mcg.dm^{-3}$)	4.37±0.48	4.18±0.34	4.54±0.63	3.83±0.23
Soluble reducing carbohydrates ($mcg.dm^{-3}$)	18.24±1.73	18.42±2.28	18.42±2.35	15.40±1.84

The results demonstrated that the biomass produced on optimized media have a better tolerance to dry-flowable formulation than biomass produced on Weidling medium, preserving better the specific activities.

Trichoderma strains produce different types of propagules, mycelial fragments, conidia, (aerial or liquid), chlamydospores, micro-sclerotia, with different bio-efficacy and/or storage stability (Mishra *et al.*, 2012). Usually, the *Trichoderma* bioproducts are made with aerial conidia, produced on moisten grains, by solid substrate fermentation (Woo *et al.*, 2014). Better survival rate and formulation stability was recently reported for chlamydospores, produced on liquid medium, containing a plant-based complex nitrogen source, i.e. cornmeal (Li *et al.*, 2016), and for microsclerotia and submerged conidia, produced on liquid media, with high carbon concentration (36 $g.L^{-1}$) and with plant-based complex nitrogen source, i.e. cotton seed flour (Kobori *et al.*, 2015).

DoE optimal medium presents important characteristics related to accumulation of more resistant propagules: the level of carbon is high and contains a plant-based complex nitrogen source. This low costs, plant-based nitrogen source, is supplemented by an additional source of growth factors and complex / organic nitrogen, yeast extract, with a proven stimulatory effect on growth and development of *Trichoderma* strains (Rossi-Rodrigues *et al.*, 2009).

The breakdown of the optimised cultivation medium ingredients costs, resulted from the techno-economic analysis is presented in figure 4. To produce 1 kg of *T. asperellum* T36 biostimulant strain the total costs for medium ingredients is 2.45 USD. More than 75% of this cost with medium ingredients is for glucose, which represent almost 81.5% from the weight of the cultivation medium ingredients.

Medium ingredients represent 28% of the total operation costs for manufacturing bioproducts based on *Trichoderma* (Klein-Marcuschamer *et al*., 2012). Thus, the cost for industrial production of biostimulant *Trichoderma* biomass is under 10 USD per kg. The value of formulation ingredients in on average on the same order. The price for the commercialisation for the field crop of such a bioproduct was considered 20 USD. kg^{-1}, with a 50% production margin (sale price double

than production costs). The treatment with a dose of 2 kg/ha of bioproduct based on biostimulant *Trichoderma* is around 40 USD/ha. The value of the additional yield resulted from such treatment on conservation agriculture systems is at least 100 USD.ha^{-1} (Oancea, 2011), which make it profitable even for field crops.

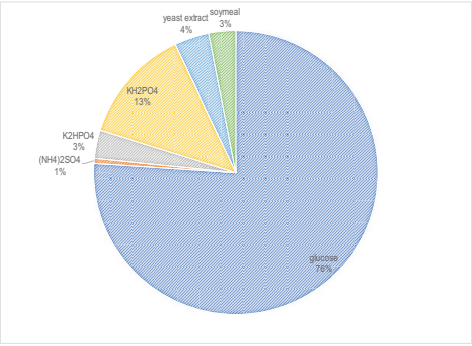

Figure 4. Breakdown of the optimised cultivation medium ingredients costs

Liquid Weidling medium contain a reach nitrogen source, bactopeptone, produced by enzymatic treatment of animal protein. Our results demonstrate that a plant-based, complex nitrogen sources, with high content on other bioactives, are more effective in promoting accumulation of fungal resting forms more resistant to formulation, than nitrogen reach animal-based product. is on agreement with others recently reported optimization of biosynthesis processes based on *Trichoderma* strains, wherein low costs meals, from oleaginous seeds, were used as complex nitrogen sources of cultivation media (Gao et al., 2013; Almeida et al., 2015).

CONCLUSIONS

We used a designed experiment, based on surface response methodology, for the optimization of medium composition for submerged biosynthesis of biomass from a biostimulant strain, *T. asperellum* T36. The studied medium components were: glucose (carbon source), ammonium sulphate (inorganic nitrogen and sulphur source), soymeal and yeast extract (organic nitrogen and growth factors source), potassium mono- and di-hydrogen-phosphates (as phosphorus source and buffering ingredients). The optimal composition of cultivation medium, resulted after experimental data analysis, is: 34.2 g. l^{-1} glucose, 0.37 g. l^{-1} ammonium sulphate, 0.8 g. l^{-1} yeast extract, 2.7 g. l^{-1} soymeal, 1.2 g. l^{-1} K_2HPO_4, 1.7 g. l^{-1} KH_2PO_4. We compared the tolerance to dry-flowable formulation of *T. asperellum* T36 biomass produced on optimized liquid media and on liquid Weidling media. The biomass produced on optimized media has a better tolerance to dry-flowable formulation than biomass produced on Weidling media. It seems that plant-based, complex nitrogen sources, with high fibres content, are more effective in promoting accumulation of fungal resting forms more resistant to formulation, than nitrogen reach animal-based products. The costs of the optimized medium ingredients determine a cost of biostimulant *Trichoderma* biomass production lower than 10 USD.kg^{-1}.

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