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TRI-SOIL®, A NEW BIOFUNGICIDE BASED ON *TRICHODERMA ATROVIRIDE* I-1237 TO CONTROL SOILBORNE DISEASES IN VEGETABLE CROP PRODUCTION

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ABSTRACT
AGRAUXINE Lesaffre Plant Care, a business unit of the LESAFFRE group, develops, produces and markets biocontrol products derived from micro-organisms to improve crop protection. Based on this expertise, AGRAUXINE developed the biocontrol product Tri-Soil®, based on *Trichoderma atroviride* strain I-1237, an antagonistic fungal micro-organism. *In vitro* studies highlighted intrinsic characteristics of this strain: strong spatial competition, development in low temperatures (5°C), strain adaptation to a broad range of pH. These features are essential to provide into the soil an effective protection of carrots against *Pythium* spp. and lettuces against *Rhizoctonia solani*. Tri-Soil® is a natural solution showing efficacy comparable to conventional chemical references for the control of soilborne diseases.

Keywords: biocontrol, micro-organism, robust, carrot/*Pythium*, lettuce/*Rhizoctonia*.

RÉSUMÉ
AGRAUXINE Lesaffre Plant Care, filiale du groupe LESAFFRE, conçoit, fabrique et met en marché des produits de biocontrôle issus de micro-organismes pour améliorer la protection des cultures. Grâce à son expérience, AGRAUXINE a développé le produit de biocontrôle Tri-Soil®, composé de la souche I-1237 de *Trichoderma atroviride*, micro-organisme fongique antagoniste. Des études *in vitro* ont mis en évidence les caractéristiques intrinsèques de cette souche : forte compétition spatiale, adaptation de la souche à la croissance en conditions froides (5°C) et à une large gamme de pH. Ces propriétés sont essentielles pour assurer au niveau du sol une protection efficace des cultures de carotte contre *Pythium* spp. et de salade contre *Rhizoctonia solani*. Tri-Soil® est une solution naturelle performante permettant d'obtenir un niveau de protection similaire à des produits conventionnels de référence.

Mots-clés : biocontrôle, micro-organisme, robuste, carotte/*Pythium*, laitue/*Rhizoctonia*.

INTRODUCTION

Soilborne plant pathogens can significantly reduce yield and quality in vegetable crops. Control of these pathogens is essential because they often survive in soil for many years and vegetable crops may be susceptible to several pathogen species. The management of soilborne diseases depends on a thorough knowledge of the pathogen, the host plant and the environmental conditions that create infection. There are different ways to fight against telluric diseases like host resistance, prophylactic measures, chemical products, biocontrol products.

Concerning used fungicides, they have a negative impact on microbial global balance in soil because they kill a wide range of fungi, pathogens or not, and cause serious damages to non-target soil microorganisms. In addition, resistances to those fungicides can reduce their efficacy. Partly for these reasons, the French plan Ecophyto was established with the objective to reduce the use of chemical products by half. In this context, methods of biological control are developed. In this approach, a product containing a viable antagonistic organism is applied to control the target pathogen.

In this study, the focus was on vegetable crops, carrot and lettuce. These crops are threatened by many soilborne pathogens, such as *Phytophthora* spp., *Rhizoctonia* spp. and *Pythium* spp. In particular, cavity spot is one of the most damaging diseases of carrot caused by a complex of several *Pythium* spp. On lettuce, the fungus *Rhizoctonia solani* is also a major telluric pathogen. In France, one active substance is registered to fight against *Pythium*-caused diseases: mefenoxam (or metalaxyl M). In the last years, few studies described limits of this treatment which started to be observed in the field : (i) repeated soil applications of mefenoxam reduce activity against cavity spot in carrots (Farrar *et al*., 2002) and (ii) resistance of *Pythium ultimum* to mefenoxam was observed in potato crops (Taylor *et al*., 2002).

Consequently, several products have been developed based on microorganisms which naturally compete with plant pathogens. For example, *Trichoderma* spp. are filamentous soil fungi that function as biocontrol agents for a large range of harmful microorganisms. The antagonistic activity of *Trichoderma* spp. is attributed to a combination of spatial and nutrient competition (Chet, 1987), mycoparasitism linked to the production of cell wall-degrading enzymes (Woo *et al*., 1999) and antibiosis (Schirmböck *et al*., 1994; Grayston *et al*., 1996). The benefits of *Trichoderma* spp. have been demonstrated for the control of soilborne pathogen diseases on several vegetable crops (Escande *et al*., 2002; Gravel *et al*., 2005; Wilson *et al*., 2008; Monteros-Barrientos *et al*., 2011; Leta and Selvaraj, 2013).

Agrauxine Lesaffre Plant Care developed and registered Tri-Soil® product, based on *T. atroviride* I1237, for its potential to protect vegetables against soilborne diseases thanks to its antagonistic activity. Firstly, *in vitro* experiments were performed in order to highlight key characteristics of *Trichoderma atroviride* I-1237 strain. Plate confrontation assays were realized to visualize spatial competition between *T. atroviride* I-1237 and *Pythium* or *Rhizoctonia* telluric pathogens. Mycelial growth assays were carried out to compare the growth rate of *T. atroviride* I-1237 and *Rhizoctonia solani*. Same tests were used to appreciate the growth capacity of several *Trichoderma* in low temperature. *In vitro* assays were also conducted to study the impact of pH on *T. atroviride* I-1237 mycelial growth. Finally, the efficacy of Tri-Soil® product (10^8 cfu/g *T. atroviride* I-1237), to control soilborne diseases was investigated in GEP field trials on carrots/*Pythium* and lettuce/*Rhizoctonia* to confirm the application rate.

MATERIAL & METHODS

IN VITRO I-1237/PATHOGEN CONFRONTATION TESTS

Plate confrontation experiments were conducted between *T. atroviride* I-1237 and the pathogen *P. ultimum* or *Rhizoctonia solani*. Pathogen strains were obtained from the collection of FREDON (Fédération Régionale de Défense contre les Organismes Nuisibles), France. Five mm PDA plugs containing *T. atroviride* I-1237, *P. ultimum* or *R. solani* were placed on PDA medium 8 cm apart in square Petri dishes (120 x 120 mm). The plates were incubated at 25°C for 7 days. Three replicates were performed per treatment.

MYCELIAL GROWTH SPEED ASSAYS

Five mm PDA plugs containing *T. atroviride* I-1237 or *R. solani* were placed on PDA medium in the middle of round Petri dishes (95 mm diameter). The plates were incubated at 25°C. Fungal mycelial growth was determined daily until the dishes were full of mycelium (2 perpendicular diameters measured per dish). Three replicates were performed per treatment. Growth speed is then calculated as the slope of the growth curve.

MYCELIAL GROWTH ASSAYS IN LOW TEMPERATURE

Five mm PDA plugs containing *T. atroviride* strain I-1237, *T. atroviride* strain SC1, *T. harzianum* strain T22, *T. gamsii* strain ICC080 or *T. asperellum* strain ICC012 were placed on PDA medium in the middle of round Petri dishes (95 mm diameter). The different strains were isolated from commercial products already used on the European market. The plates were incubated at 5°C. Fungal mycelial growth was then determined regularly, at least one time per week (2 perpendicular diameters measured per dish). Three repetitions were performed per treatment.

IMPACT OF PH ON I-1237 MYCELIAL GROWTH

Mycelial plugs (4 mm) containing *T. atroviride* I-1237 were deposited in the middle of Petri dishes (95 mm diameter) containing 15 ml of PDA medium at different pH values (3, 4, 5, 6, 7, 8 and 9). Petri dishes were incubated at 20°C. Fungal growth was determined daily (day 1 to day 4) by radial measures of the mycelial colonies. Measurements of mycelial growth were done for five Petri dishes for each pH values. Three radial measures were determined per Petri dish.

TRI-SOIL® EFFICACY IN CARROTS AND LETTUCE IN FIELD TRIALS

Field trials carrots/*Pythium* spp.

The efficacy of Tri-Soil® was calculated on % incidence of carrots infected, compared to the Untreated Control (UTC). The average incidence was calculated from data of 14 field trials (13 GEP) performed in France, Germany and Netherlands between 2007 and 2015. General guidelines were EPPO PP 1/152 (4), EPPO PP 1/181 (4) and EPPO PP 1/135 (4). Specific guidelines were EPPO PP 1/148(2) and CEB 223. Plot size was 9-36 m² and 4 replicates* were realized (* 2 replicates in the non GEP trial). Tested varieties were Rodelika, Bentley, Rote Riesen 2, Nevis, Narbonne F1, Nairobi, Naval, Maestro and Champion. Tri-Soil® was applied once at 5 kg/ha by spraying soil (200-600 L/ha water volume) 0 to 3 days before sowing. The conventional chemical treatment (REF) was mefenoxam or propamocarb (1 trial) applied one or two times at 0,5 L/ha (registered dose) at BBCH 11 & 18-42. Assessments were % of infected carrots (incidence).

Field trials lettuce/*Rhizoctonia* spp.

The efficacy of Tri-Soil® was calculated on % incidence of lettuce infected, compared to the Untreated Control (UTC). The average incidence was calculated from data of 8 field trials (5 GEP) performed in France, Germany and Italy between 2007 and 2015. General guidelines were EPPO PP 1/152 (4), EPPO PP 1/181 (4) and EPPO PP 1/135 (4). Specific guidelines were CEB 110 & 130. Plot size was 5-12 m² and 4-5 replicates were realized. Tested varieties were Civezza, Lobelia, Batavia Tourbillon, Lucant, Romane, Dragonne SV 1065 LA and Paola Almagro. Tri-Soil® was applied once at 5 kg/ha by spraying soil (150-400 L/ha water volume) 1 to 3 days before sowing. The conventional chemical treatment (REF) was pencycuron (20 L/ha for soil application, 3 L/ha for foliar application) or propamocarb (0,3 L/ha) or pyraclostrobin + boscalid (1,5 kg/ha) applied 1 to 3 times from 1-2 days before sowing to BBCH 45. Assessments were % of infected lettuces (incidence).

RESULTS

TRICHODERMA ATROVIRIDE I-1237 IS A ROBUST ANTAGONISTIC STRAIN AGAINST TELLURIC PATHOGENS

I-1237 antagonistic activity thanks to the combination of several modes of action

The antagonistic activity of *T. atroviride* I-1237 is notably linked to a strong spatial competition. Indeed, *in vitro* confrontation experiments on Petri dishes showed that I-1237 remarkably reduced the mycelial growth of *Pythium ultimum* (Figure 1A, B) and *Rhizoctonia solani* (Figure 1C, D) pathogenic fungi. Moreover, we can observe that I-1237 overgrew the colonies of *P. ultimum* and *R. solani* on the Petri dishes. I-1237 property to invade space is related to its capacity to grow very quickly, faster than the pathogen *R. solani* for example (Figure 2).

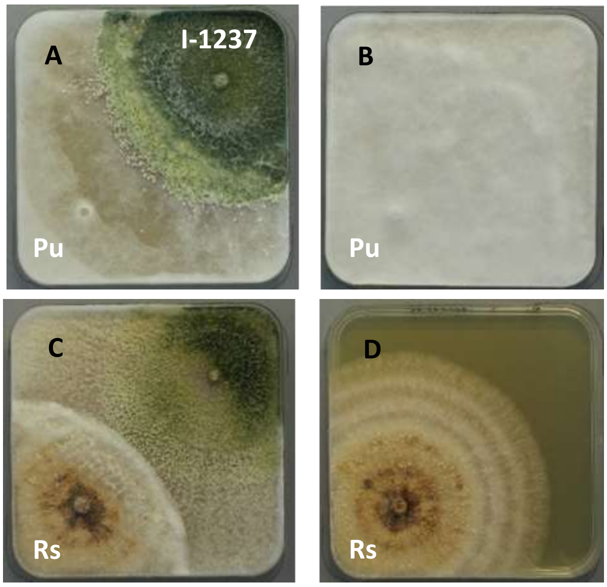


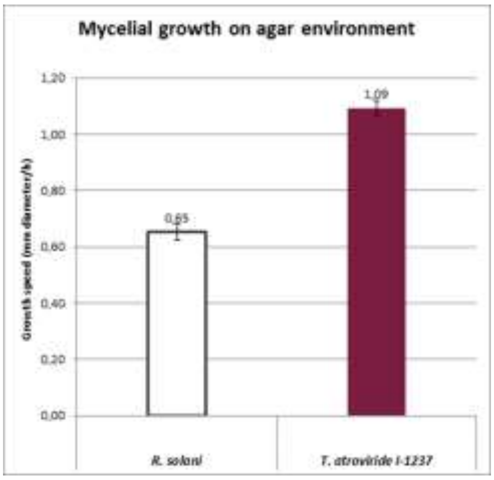
Figure 1 : *In vitro* confrontation tests between *T. atroviride* I-1237 and *Pythium ultimum* (**A**, **B**) or *Rhizoctonia solani* (**C**, **D**). **B**, **D**: pathogen alone; **A**, **C**: pathogen and I-1237 in co-culture.

Figure 1 : Tests de confrontation *in vitro* entre *T. atroviride* I-1237 et *Pythium ultimum* (**A**, **B**) ou *Rhizoctonia solani* (**C**, **D**). **B**, **D**: pathogène seul; **A**, **C**: pathogène et I-1237 en co-culture.

Pu = *Pythium ultimum*

Rs = *Rhizoctonia solani*

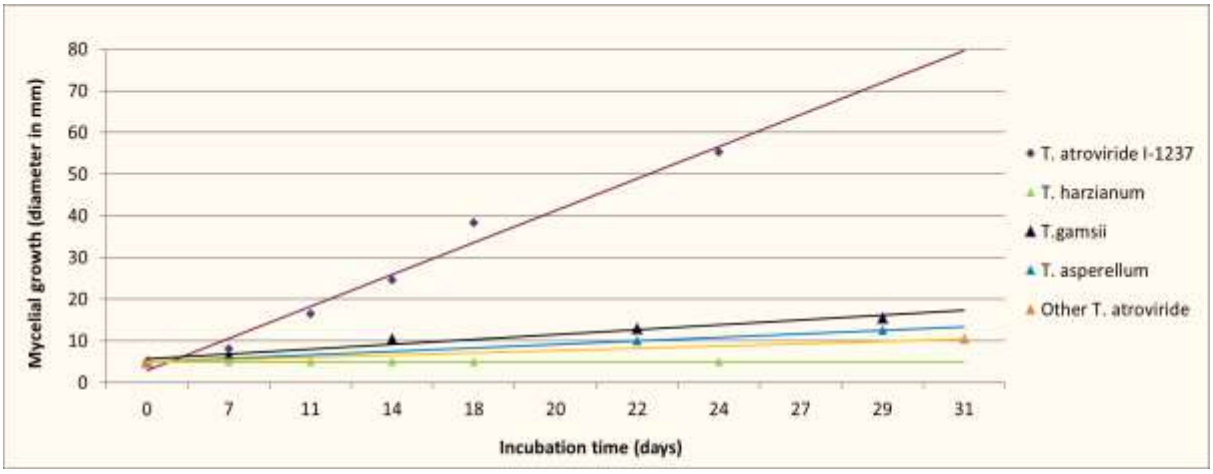
Figure 2 : Mycelial growth speed of *T. atroviride* I-1237 compared to *Rhizoctonia solani* on PDA medium at 25°C.
Figure 2 : Vitesse de croissance mycélienne de *T. atroviride* I-1237 comparée à *Rhizoctonia solani* sur milieu PDA at 25°C.



Development of I-1237 in low temperatures

Laboratory *in vitro* tests highlighted the very good capacity of *T. atroviride* I-1237 to grow in low temperatures. Compared to other *Trichoderma* species or other strain of *T. atroviride*, I-1237 mycelial growth at 5°C was 3 to 6 times faster (Figure 3).

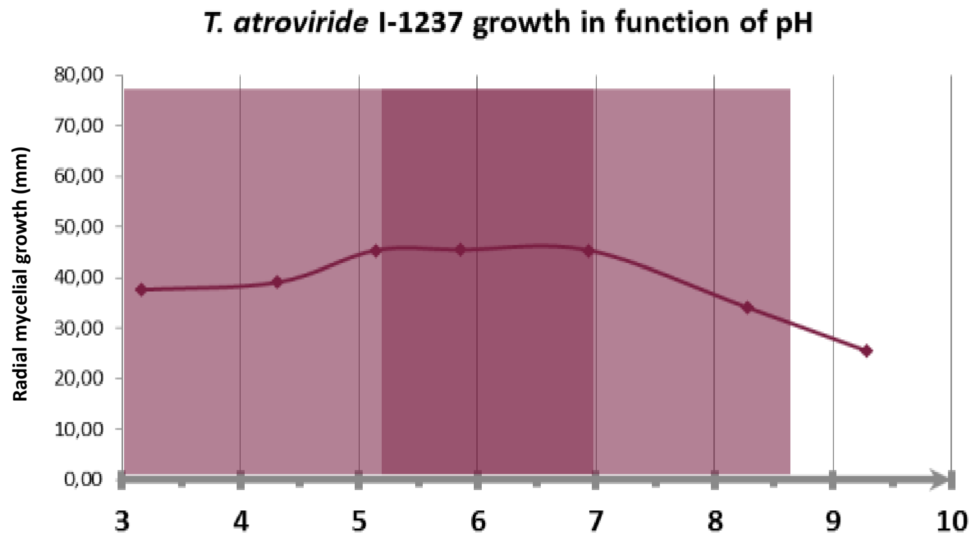
Figure 3 : Mycelial growth at 5°C of *T. atroviride* I-1237 compared to other *Trichoderma* on PDA medium.
Figure 3 : Croissance mycélienne à 5°C de *T. atroviride* I-1237 comparée à d'autres *Trichoderma* sur milieu PDA.



I-1237 is adapted for acidic and basic pH

In vitro studies showed that *T. atroviride* I-1237 is adapted to acidic and basic pH, which did not strongly disturb the mycelial development of the fungus (Figure 4). Similarly, the germination of I-1237 spores was not affected when suspended for 4 hours in water solution with a broad range of pH (3 to 9) (data not shown).

Figure 4 : Mycelial development of *T. atroviride* I-1237 with regard to pH value of the culture medium (PDA).
Figure 4 : Croissance mycélienne de *T. atroviride* I-1237 en fonction du pH du milieu de culture (PDA).

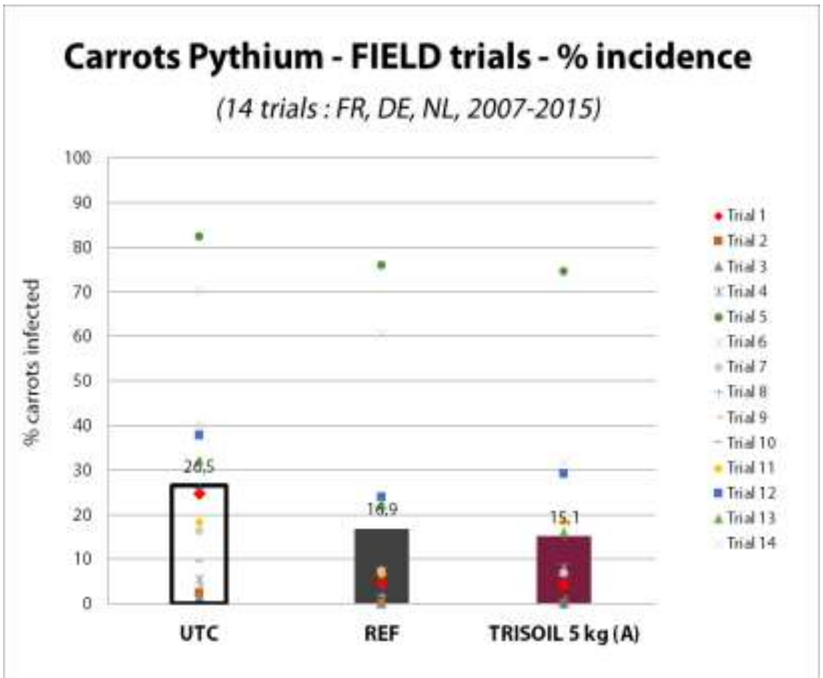


EFFICACY OF TRI-SOIL® DEMONSTRATED IN GEP FIELD TRIALS ON CARROT AND LETTUCE AGAINST SOILBORNE DISEASES

Field trials carrots/*Pythium* spp.

Compared to UTC, the average efficacy of Tri-Soil® in 14 trials was satisfactory (55%). Indeed, the number of infected carrots (incidence) for the UTC was 26.5% whereas 15.1% for Tri-Soil® treatment (Figure 5). For the conventional chemical reference, results are comparable to Tri-Soil® treatment with 16.9% of infected carrots.

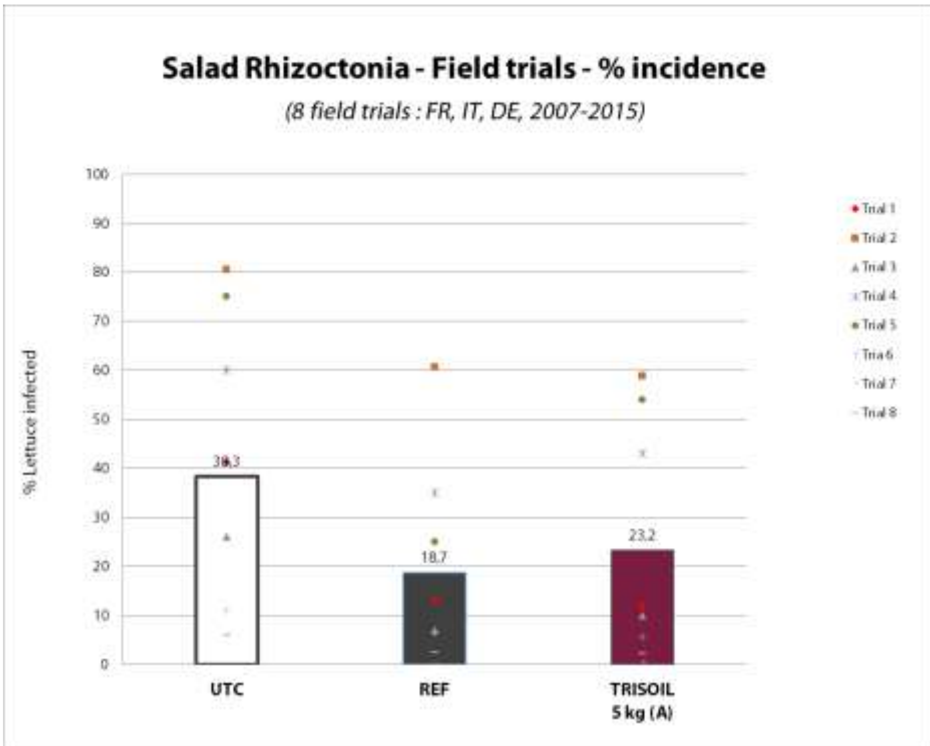
Figure 5 : Efficacy of Tri-Soil® on *Pythium* spp. in carrots: average percentage of disease incidence on crop treated with Tri-Soil® or a conventional chemical treatment (REF) in 14 field trials from 2007 to 2015. Results obtained in each trial are also represented. UTC = Untreated Control.
Figure 5 : Efficacité de Tri-Soil® sur *Pythium* spp. en culture de carotte: pourcentage moyen d'incidence de la maladie sur cultures traitées avec Tri-Soil® ou une référence chimique (REF) dans 14 essais au champ entre 2007 et 2015. Les résultats obtenus pour chaque essai sont également représentés. UTC = Témoin non Traité.



Field trials lettuce/*Rhizoctonia* spp.

Compared to UTC, the average efficacy of Tri-Soil® in 8 trials was satisfactory (50%). Indeed, the number of infected lettuces (incidence) for the UTC was 38.3% whereas 23.2% for Tri-Soil® treatment (Figure 6). For the conventional chemical reference, results are comparable to Tri-Soil® treatment with 18.7% of infected lettuces.

Figure 6 : Efficacy of Tri-Soil® on *Rhizoctonia* spp. in lettuce: average percentage of disease incidence on crop treated with Tri-Soil® or a conventional chemical treatment (REF) in 8 field trials from 2007 to 2015. Results obtained in each trial are also represented. UTC = Untreated Control.
Figure 6 : Efficacité de Tri-Soil® sur *Rhizoctonia* spp. en culture de laitue: pourcentage moyen d'incidence de la maladie sur cultures traitées avec Tri-Soil® ou une référence chimique (REF) dans 8 essais au champ entre 2007 et 2015. Les résultats obtenus pour chaque essai sont également représentés. UTC = Témoin non Traité.



DISCUSSION

In vitro tests revealed advantageous characteristics of *T. atroviride* strain I-1237: antagonistic activity linked to a strong spatial competition, fast growth, very good capacity to grow in low temperatures, adaptation to acidic and basic pH.

Concerning the action mode of *T. atroviride* strain I-1237, previous experiments demonstrated that its antagonistic activity is also associated to antibiosis (Mounier *et al.*, 2015a). Thus, Tri-Soil® benefic activity against soilborne diseases is due to the combination of several modes of action.

The ability of *T. atroviride* I-1237 to develop in low temperature is essential for an active substance intended to be applied on vegetable crop soil. Indeed, soil temperatures can be frequently fresh, even cold, in sowing or harvest periods, especially in carrot crop. *T. atroviride* I-1237 is also the active substance of Esquive®WP product, registered by Agrauxine for the control of grapevine trunk diseases such as Esca and BDA (Mounier *et al.*, 2015b). Applied on pruning wounds during the winter, the capacity of I-1237 to grow in low temperature is crucial for its penetration and implantation into the wood. The use of *T. atroviride* I-1237 for soil or wood treatment underlines the adaptability of this *T. atroviride* strain. Spore germination of *T. atroviride* I-1237 was not affected by pH, highlighting the robustness of this strain. Indeed, spore germination of several fungal species is inhibited by acidic or basic pH, as for example *Penicillium expansum* (Li *et al*., 2010).

CONCLUSION

Laboratory experiments showed that the *T. atroviride* strain I-1237 is a robust strain, able to develop in low temperature conditions and with a broad range of pH. *T. atroviride* strain I-1237 capacity of fast growth is crucial for its benefic antagonistic activity.
Formulated into Tri-Soil® product (10^8 cfu/g) used at 5 kg/ha in preventive soil treatment, *T. atroviride* strain I-1237 was demonstrated in GEP field trials to protect efficiently vegetable crops against soilborne diseases such as *Pythium* in carrots and *Rhizoctonia* in salads. Moreover, efficacy levels observed in field were very satisfactory since Tri-Soil® displayed the same efficacy than chemical references.
Tri-Soil® registration in France was obtained by Agrauxine in November 2016. Based from a natural active substance, Tri-Soil® is a product with no-LMR profile and is compatible with organic farming.

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