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Advances in Our Understanding of the Disease Biocontrol Potential and Enhancement of *Trichoderma harzianum* Strain T22 (Trianum®)

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Abstract

The commercial product Trianum-P is a registered plant protection product for the control of plant diseases in growing media. It contains the *Trichoderma harzianum* strain T22 which is arguably the most widely applied beneficial fungus in commercial horticulture today (Harman et al., 2004a). As part of this year's Trianum-P development work, the author carried out a series of small-scale tests and trials to evaluate the potential benefits of combining Trianum-P with various commercial biostimulants in the production of ornamental plants. One of the challenges of using disease biocontrol agents in crop production is an apparent inconsistency in field performance. It is incumbent on us to find ways to enhance the disease biocontrol activities of T22 and other disease antagonistic micro-organisms. The results obtained in this preliminary work have shown that the combined treatments of Trianum-P and various biostimulants produced various plant growth effects and reduced disease incidence. Fungal biomass development of *T. harzianum* (T22) was favoured by 0.05% Vidi Parva and the pelleted plant-based product Vidi Funda. The biostimulant Fortafol-D inhibited *T. harzianum* conidiospore germination at the commercial rate (0.1%v/v). The significance of this is discussed. Early plant growth stimulation effects by Trianum-P were observed in *Tagetes*. *Lavandula* plugs treated with Trianum-P + Vidi Parva developed less root disease incidence and more shoot extension growth compared to untreated controls. In *Ceanothus* the Trianum-P treatment produced the best overall plant shape and more spring growth than all other treatments. A reduced number of flower heads in the Trianum-P treated *Ceanothus* may have been attributed to stress reduction effects and growth hormone induction resulting from *T.harzianum*'s symbiotic

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associations with the plant's roots. In *Hebe* the greatest root biomass was produced by the Trianum-P + Vidi Parva and M149 + Vidi Parva treatments. Trianum-P + Vidi Parva gave the best results overall, scoring highly in root biomass production, strong lateral root production and achieving the greatest reduction in disease incidence. In *Deutzia* more root biomass and the best root quality was produced in the Trianum-P and Trianum-P + Vidi Parva treatments.

INTRODUCTION

Trichoderma harzianum strain T22 was generated by combining protoplast tissue from *Trichoderma harzianum* strains T95 and T12 (Stasz *et al*., 1988) and is now sold commercially as the product Trianum. The development of T22 marked the beginning of a new commercial era in disease biocontrol around the World. Although there are two commercial formulations available, this paper will focus on the powdered formulation Trianum-P. This has several label and off-label approvals for use in ornamentals, soft fruit and salads (Anon, 2019a). Additional product literature is available at www.Koppert.co.uk (Anon, 2019b, 2019c).

In early studies both of T22's parent isolates T95 and T12 demonstrated strong disease biocontrol activity and rhizosphere competence (Ahmad & Baker, 1988). The commercial production of Trianum is carried out under clinical conditions in computer controlled stainless steel fermenter vats. The computerized system ensures that the precise biotic and abiotic requirements are provided so that optimal conidiospore to biomass ratios are achieved. As with plants, the precise nutritional needs of T22 must be met in order to generate the necessary high-quality yields in a cost-effective manner.

Mechanisms of action

Gradually scientific research has unraveled the complex methods employed by T22 to gain a competitive advantage over disease-causing plant pathogenic fungi. Different effects and observations pointed to multiple mechanisms of action including mycoparasitism, antibiosis, a high competitive saprophytic ability, the production of pathogen cell wall degrading enzymes, ability to colonise the roots of plants (rhizosphere competence) and the ability to induce systemic resistance in plants. When a pathogen is under attack, the T22 hyphae coil around its host, and produce chitinase and β-1,3 glucanase enzymes which produce scars, pits and perforations in the pathogen hyphal wall causing leakage of cellular contents (Harman *et al*., 2004a; Harman, 2006). T22 produces secondary metabolites in artificial culture and under natural conditions in the plant's rhizosphere at concentrations which are fungitoxic to pathogens (Hanson, 2005; Vinale *et al*., 2006; Harman, 2006). T22 is rhizosphere competent and occupies the root surface preventing fungal plant pathogens access to the root surface or infection court.

The biocontrol agent is also able to penetrate root epidermal cells causing the production and release of a variety of compounds that lead to induced systemic resistance and pathogen suppression on the shoot remote from T22's original point of entry on the root (Heil & Bostock, 2002). Root application of *T. harzianum* strain T22 reduces foliar infections in tomato plants against cucumber mosaic virus (Vitti *et al*., 2016) and *Alternaria solani* (Harman *et al*., 2004a). Maize seeds treated with T22

increased plant resistance against foliar anthracnose caused by *Colletotrichum graminicola* (Harmen *et al*., 2004b).

Nursery trials and experiments
Vidi Parva and T22 spore germination

Trianum-P can be applied with Vidi Parva to provide root growth benefits and a disease prevention measure at the same time. Vidi Parva is a 100% natural plant-based biostimulant which is manufactured using cold compressed seaweed extracts from *Ascophyllum nodosum*. The L-tryptophan content of Vidi Parva is a pre-cursor amino acid involved in the production of plant auxins (Zhao, 2012). Specific auxins stimulate the formation of root branches and root hairs and supports the development of a strong plug plant with a well-developed root system (Nibau, 2008).
A static culture experiment was carried out on three separate occasions to determine if Vidi Parva can act as a source of nutrition and directly benefit the growth of T22 mycelium. The assessment was based on a visual estimate of the percentage volume of solution that was occupied by T22 mycelium. The most concentrated solution was prepared by adding 5 ml of Vidi Parva to 95 ml of tap water in 150 ml clear plastic bottles, and this was used to perform a 100-fold serial dilution to produce seven Vidi Parva dilutions in total. There was 100 ml of solution per container and each solution was inoculated with 0.05 g of Trianum-P. The containers were sealed with a lid and maintained at room temperature (18-21^{o}C) for 7 days. The 0.05% test dilution was the closest concentration that matched the recommended application rate for Vidi Parva (1-2ml/m^2 in 1-2 litres of water) in commercial plant propagation situations.

T22 spore germination was evident after 2 days in all solutions except the most concentrated solution (5%). The 0.05% solution produced more biomass within the first two days than any other solution. In the weakest solutions ($5x10^{-10}$ and $5x10^{-12}$) the small quantity of mycelium was similar to the water control. The results suggest that the commercial rate used for root development purposes could feasibly promote the development of T22 when used together in a plant production environment.

Trianum-P + Vidi Funda

The activity of resident disease antagonistic micro-organisms in soils can be stimulated by the addition of organic amendments such as farmyard manure, green manures and straw. These materials are freely available but vary in composition. In early product development work, the high quality manufactured pelleted organic amendment Vidi Funda also increased the growth of various beneficial fungi. Therefore, the product was tested as a potential 'solid-substrate partner' for Trianum-P. The product is sold as a soil conditioner and organic fertiliser delivering N, P and K (7:2:4) over 4-12 weeks.
An experiment was set up to compare six treatments. There were three replicates per treatment. Each container (11x11x5.5 cm) consisted of 20 g of Klasmann bedding compost and either 2 g of Vidi Funda (low rate) or 10 g of Vidi Funda (high rate). Containers were either treated with 2 g Trianum-P, 2 g M149 (a coded trial product containing *Trichoderma spp*) or remained untreated. All containers received 15 ml tap water and were lightly mixed with a spatula. The lidded containers were incubated at room temperature and checked for fungal colonisation over 28 days. The biomass produced by M149 and Trianum-P was increased at both rates of Vidi Funda, compared to untreated controls, but more biomass was produced at the high rate. This experiment demonstrated that the colonisation of non-sterile growing media by Trianum-P was increased by the addition of Vidi Funda.

Trianum-P + Fortafol

Fortafol is a biostimulant which is formulated as a water-soluble emulsion and contains humic and fulvic acids, and plant extracts from *Thymus* and *Mentha*.

Since it is feasible that Fortafol and Trianum-P could be used as part of the same integrated crop management programme, it was necessary to test the compatibility between the two products. Previously it has been shown that the viability of fungal spores of various plant pathogens are rendered non-viable following exposure to a Fortafol solution. Further proof and clarity regarding the compatibility between Fortafol and strain T22 was required.

The effects of Fortafol, Switch and Cercobin on T22 spore germination were tested at five concentrations ranging from 0 to 1% v/v in a 100-fold serial dilution. To each dilution 0.05 g Trianum-P was added to 100 ml of the diluted solution in 150 ml clear plastic bottles with sealable caps. The pots were placed in the dark in a box at room temperature (16-20^{0}C).

In the 1% and 0.01% solutions of Fortafol, Switch and Cercobin, the spores of T22 failed to germinate after 14 days, but spore germination and mycelium development did occur in the weak solutions ($1x10^{-4}$ and $1x10^{-6}$) and water control. The recommended application rate for Fortafol as a substrate treatment is 1ml/l (0.1%v/v). Since no germination occurred at 1% and 0.01%, it is reasonable to assume that germination would not occur at the commercial rate either. This experiment confirms that Fortafol-D must not be applied as a tank mix. The current advice for a growing media drench or drip line treatment is to apply Trianum-P first followed by Fortafol-D three days later.

Trianum-P & plant growth promotion in *Tagetes*

The plant growth promoting effects of *Trichoderma* are well documented (Baker, 1988; Ousley, *et al*., 1994). An experiment was set up to compare two Trichoderma species as growth promotors of marigold (*Tagetes sp*).

A total of 60 marigold seeds were sown in Klasmann bedding plant substrate in polystyrene trays in early March. There were two trays per treatment and 10 seeds per tray. The trays were watered, covered with a plastic film until seedling emergence and placed in a cold glasshouse with frost protection (minimum temperature of 5^{o}C). After 7 days all seeds had germinated and had reached the cotyledon stage. Trianum-P and M149 were applied as a compost drench after 4 days. The untreated control seedlings were treated with water. Trianum-P and M149 were applied at 1.5 g/l of water and each cell received 5 ml of solution. A second treatment was applied 10 days later at a rate of 0.5 g per cell followed by irrigation to move the dry product into the compost profile.

Assessments were made 15 days and 24 days after the first treatment. Growth differences between treatments became evident from the cotyledon stage. After 15 days, the mean stem height, mean cotyledon length and mean length of the first true leaves were determined. The largest growth differences occurred in the Trianum-treated seedlings, followed by the water-treated control. The smallest seedlings were produced in the M149 treatment. After 24 days the stem height difference between water and Trianum-treated seedlings had narrowed and was no longer significantly different. The most significant difference occurred in the first true leaves, measuring mean lengths of 47.1 mm (18.5% increase), 40.4 mm and 36.2 mm (10.4% decrease) for Trianum-P, untreated and M149 respectively.

Lavandula hidcote propagation trial

Very few studies have been carried out to evaluate the combination of Trianum-P with the rooting stimulant Vidi Parva, in the propagation of hardy ornamental nursery stock.

A *Lavandula* propagation trial compared the treatment combinations of Trianum-P + Vidi Parva (TP) and M149 + Vidi Parva (MP) against an untreated control (U). Vidi Parva was included due to its positive effect on T22 spore germination and growth as reported earlier in this paper. Due to the limited number of *Lavandula* cutting trays available for this trial, it was not possible to include solo treatments of Trianum-P, M149 or Vidi Parva.

The nursery had previously experienced a high disease incidence in *Lavandula* leading to significant losses, and there were also three-week-old diseased *Lavandula* cuttings in an adjacent area when the trial was set up. There was also an unprotected period of 7 days between the sticking date and treatment date.

There were 12 trays of cuttings with four trays per treatment. There were 60 cells per tray and each tray measured 45cm x 27 cm. The cuttings were prepared on 26th April and the treatments were applied on 3rd May. Trianum-P and M149 were applied at the rate of 2.5 g/m^2 and Vidi Parva at 5 ml/m^2 in a water volume of 2 l/m^2. The cuttings were maintained on a sand bed and misted frequently to maintain humidity. After six weeks there was significantly (P=0.05) less disease incidence in the TP treated plugs compared to untreated ones. Other treatment mean comparisons yielded no significant differences.

Trianum-P growth effects in nursery stock

A commercial trial was set up to study the growth effects of Trianum-P in *Deutzia nikko*, *Hebe* x *andersonii* 'Variegata' and *Ceanothus repens*. On close examination some of the *Deutzia* roots were discoloured and collapsed caused by soil borne disease infections. The *Hebe* roots were also discoloured but this was considered normal for this plant species. The *Ceanothus* roots were white and healthy. A total of 126 plugs were potted-on into 9 cm pots (volume 570 cm^3) in Klasmann container substrate on 26th March. The trial consisted of seven treatments and 18 plants per treatment (three plant species x six plant replicates per treatment). All pots received two treatments, the first on the 1st April and the second on the 2nd May. After potting-on all 126 pots received 1 g of Vidi Funda (7:2:4) which was applied to each pot individually to provide plants with equal levels of fertiliser for the three-month trial period. The treatments were:

1) Untreated
2) M149 (2g/m^2)
3) Trianum-P (2g/m^2)
4) Vidi Parva (2.8 ml/m^2)
5) M149 (2g/m^2) + Vidi Parva (2.8 ml/m^2)
6) Trianum-P (2g/m^2) + Vidi Parva (2.8 ml/m^2)
7) Fortafol (2.8 ml/m^2)

Treatment solutions were applied at a rate 100 ml per pot when the growing substrate was beginning to dry. The untreated pots received an equivalent volume of plain water. The target rates were in line with the recommended rates: Trianum-P – 15 g/1000 pots, Parva – 3 l/ha, Fortafol – 3 l/ha. The trial product M149 was also applied at the rate of 15 g/1000 pots.

A. *Ceanothus repens*: The assessments carried out on the 8th May (37 days after the first treatment) identified a number of growth differences. There was a statistically significant (P=0.05) increase in the mean lateral length produced in Fortafol-treated *Ceanothus* plants, when compared with the Vidi Parva and water treatments, but not Trianum-P solo, or M149 solo, or

combination treatments. There was no difference (P=0.05) between the number of lateral shoots produced in all treatments. On the 11th May (41 days after the first treatment) there were 72%, 44.4%, 33.3%, 27.8%, 27.8% and 27.8% fewer open flower heads in the Trianum-P, Fortafol, M149, Vidi Parva, Trianum-P+ Vidi Parva and M149 + Vidi Parva treatments respectively, compared to the control. On 7th June the Trianum-P treated plants were rated as being the best quality (based on leaf colour, number and leaf area), having the best shape (good branching; moderate gap between leaves) and having the greatest amount of spring growth overall (plant height, vegetative growth).

B. *Hebe* x *andersonii* 'Variegata': There were significantly more shoots produced in the Trianum-treated *Hebe* plants compared with the untreated control at the 5% probability level. There were no significant differences between all other paired treatment means. An assessment of root growth was carried out on the 12th May (41 days after the first treatment). Compared to the untreated plants, the greatest root biomass was produced in the Trianum-P + Vidi Parva and M149 + Vidi Parva treated plants. These treatments also produced the healthiest looking roots. Blackened disease-infected roots were present in all treatments with the lowest incidence in Trianum-P + Vidi Parva and Trianum-P solo treated plants. Root lateral branching occurred in all Vidi Parva treatments (combinations and solo) and Trianum-P solo treated roots, but lateral branching was low in Fortafol, M149 and untreated plants.

C. *Deutzia nikko*: An assessment of root growth was carried out on the 9th June (69 days after the first treatment). Compared to the untreated plants, the greatest root biomass and best root quality was produced by the Trianum-P + Vidi Parva and Trianum-P treatments.

Conclusion and Discussion

The trials demonstrated that Trianum-P can stimulate the growth of different plant species raised from seeds (*Tagetes*), cuttings (*Lavandula*) and plugs (*Deutzia*, *Ceanothus and Hebe*). The application of Vidi Parva as a tank mix partner can also contribute to growth and health improvement in *Hebe*, *Deutzia* and *Ceanothus*. This work provided some important evidence to support the use of Trianum-P and Vidi Parva in future nursery stock production for plant growth and development and disease prevention purposes. Vidi Parva yielded an increase in T22 growth in artificial culture suggesting that Trianum-P was able to utilise Vidi Parva as a source of nutrition. The provision of high (specific) levels of nutrition under natural growing conditions could be important for the biocontrol activities of mycoparasitism (enzyme production) and antibiosis (secondary metabolite production) (Jackson *et al*., 1991a). The growth of T22 was also supported by the plant-based pelleted fertiliser Vidi Funda. It is therefore feasible that this food source could also enhance T22's disease biocontrol activities. It might also favour T22's longer-term survival prospects and greater potential for self-perpetuation (Jackson *et al*., 1991b). It was confirmed that Fortafol is fungitoxic to T22 spores at the commercial rate and must not be applied as a tank mix. This preliminary work has given an insight into how biostimulants and Trianum-P could be used together in future crop management matrices.

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