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D9.6
Recommendations of BCAs treatments after evaluation in field experiment

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1. Summary

This deliverable draws on two major tasks within WP9 of PURE, firstly, the development of BCAs active against plant pathogens (Task 9.2, led by Ilaria Pertot, FEM) and, secondly, the development of BCAs active against insect pests (Task 9.3, led by Emilio Guerrieri, CNR). Notable outputs included:

1. An improved formulation to enhance germination of *Ampelomyces quisqualis*, a hyperparasite of powdery mildew. This resulted in an increase in efficacy of up to 20% against powdery mildews of cucurbits, grape and strawberry.
2. Development of formulation substrates to increase the survival of *Trichoderma atroviride*, a BCA active against soil-borne plant pathogens.
3. Characterization of BCAs active against the tomato leafminer, *Tuta absoluta*, out of which the most promising was the egg parasitoid, *Trichogramma achaeae*.
4. Development of rearing protocols to improve the fitness and parasitization efficiency of *T. achaeae* against *T. absoluta*. Fitness, in terms of longevity and fertility, was increased for parasitoids reared on *Tuta absoluta* eggs and BCA performance efficacy was improved by using appropriate acclimation temperatures. We found a positive correlation between temperature during acclimation and at oviposition so that cold acclimated specimens performed better at cold temperatures and warm acclimated ones performed better at warm temperatures.

Thus, the improved formulation of *Ampelomyces quisqualis* is recommended for control of powdery mildew and *Trichoderma atroviride* with the appropriate (shrimp shell) substrate is recommended for control of soil-borne plant pathogens. Efficacy of these treatments is improved if they are applied earlier in the season. *Trichogramma achaeae*, reared under the appropriate conditions, is recommended for control of tomato leafminer. *T. achaeae* mass releases on their own are not sufficient and use in combination with other biological control methods (mirid predators and mating disruption) is recommended. *T. achaeae* should be released at the onset of tomato-growing cycle, when other antagonists of *T. absoluta* are not yet well established in tomato fields.

2. Objectives

- To test new innovative *A. quisqualis* treatments against powdery mildews of apple (for WP5), grapevine (for WP6) and greenhouse crops as strawberry and cucurbits (for WP7).
- To test the role of organic substrates to increase longevity of BCAs for use against soil-borne diseases as *Rhizoctonia solani* on potatoes (for WP4), *Rosellinia necatrix* on apple (for WP5) and *Armillaria mellea* on grapevine (for WP6).
- To test the effect of *T. achaeae* rearing system on its efficacy as a BCA against *T. absoluta* (for WP 7).
- To test if preimaginal thermal acclimation influences parasitism rate of *T. achaeae* on *T. absoluta* (for WP 7).

3. Deliverable procedure

3.1 To test new innovative *A. quisqualis* treatments against powdery mildews of apple, grapevine and greenhouse crops as strawberry and cucurbits.

Ampelomyces quisqualis is a hyperparasite of powdery mildew and the active substance of one of the oldest commercial biofungicides (AQ10). Once applied on the powdery mildew infected leaves, *A. quisqualis* conidia germinate and hyphae penetrate mycelium of the powdery mildew host. After penetration, it continues its growth internally and suppresses both asexual and sexual sporulation of the attacked powdery mildew mycelia by colonizing and destroying the conidiophores and the immature fruiting bodies. Under field conditions *A. quisqualis* efficacy in controlling powdery mildew is reported to be inconsistent. This is often related to unsuitable environmental conditions in which the conidia are exposed before penetration. Conidia are negatively affected by UV irradiation, low relative humidity and high temperatures. The conidia of *A. quisqualis* poorly germinate in absence of the host, and the germination rate and speed is strongly promoted by an unknown substance produced by powdery mildew.

We developed an additive to be combined with *A. quisqualis* strains (6 hours pre-germination) to mimic the presence of powdery mildew and speed up the germination process in order to reduce the time in which conidia may be exposed to adverse conditions. This resulted in an increase in efficacy of up to 20% against powdery mildews of cucurbits, grape and strawberry (full data are being published and will be made available after publication). The efficacy against overwintering inoculum of grapevine powdery mildew (chasmothecia) was not increased by the pre-germination with the additive, most probably because at the time of application in the field (late summer/beginning of autumn) the environmental conditions were already suitable for parasitization by *A. quisqualis*.

The main recommendations and conclusions were:

- Different substances have been tested in term of promotion of germination and enhancement of germ tube growth of A. quisqualis. The best performing substance was a water extract of shrimp shells.
- The best conditions and dosage for pre-germination of A. quisqualis conidia have been identified. Different strains of A. quisqualis have different levels of aggressiveness towards the powdery mildew, however the substance seems to activate all of them (nonspecific activity).
- The pre-germination treatment was tested under commercial-like conditions against powdery mildew of crops included in WP 5 (apple), WP6 (grapevine) and WP7 (cucurbits and strawberry) in order to obtain an indication of the activity. In all patho-systems the pre-germination treatment resulted in an increase in efficacy. However, there was no increase in efficacy when the formation was tested against chasmothecia of Erysiphe necator on grapevine, most probably because the environmental conditions in late summer-early autumn were already suitable for the A. quisqualis activity.
- The recommendation for the use of A. quisqualis is to apply it only if some signs of the disease are present (under field conditions in the absence of powdery mildew mycelium, the antagonist dies quickly) and to apply the treatment only when the environmental conditions are suitable for A. quisqualis survival and parasitization process. Pre-germination treatment with a germination-inducing substance is recommended because it can improve the efficacy of A. quisqualis. The pre-germination treatment did not result in any phytotoxicity against the plant.

3.2 To test the role of organic substrates to increase longevity of BCAs for use against soil-borne diseases as *Rhizoctonia solani* on potatoes, *Rosellinia necatrix* on apple and *Armillaria mellea* on grapevine.

Trichodermas are known to be good antagonists against soil pathogens, but their survival in the soil is often too short to reach full efficacy. However, they can easily colonize lignin, cellulose and chitin-rich substrates, which are slowly degraded in the soil, and these can increase their survival. Some organic substrates can thus be infiltrated with BCAs to enhance trichoderma survival in the soil. Different organic supports, which are available at low cost as by-products of the wood industry or tannin extraction, or fish farms were evaluated (barks, wood chips of different type and shrimps shells). Pre-treatment with bark mixtures (fir, larch and pine) or a by-product of shrimp farms (shrimp shells) increased the survival of *Trichoderma atroviride* SC1 (use as a reference strain) in soil. The inoculation of barks either reduced the risk of contamination with *Armillaria mellea* (when barks were infected by this pathogen; Fig.1) or reduced the incidence of the diseases when the soil was already infected (Table 1).

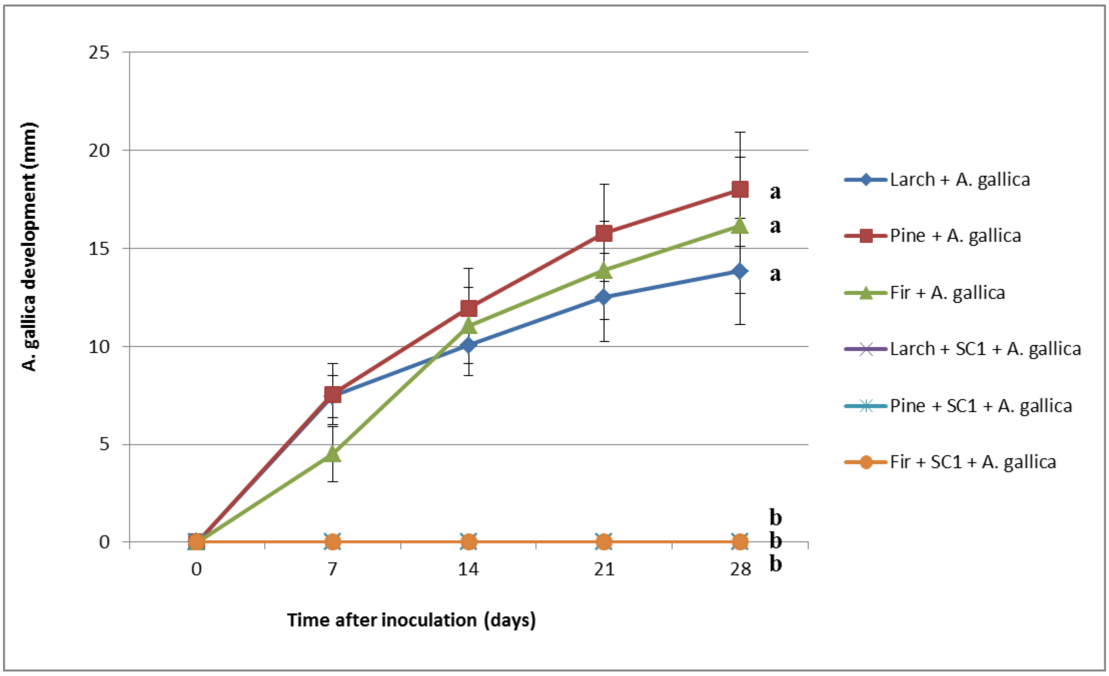

Fig.1 Reduction of *Armillaria gallica* development on barks pre-treated with *Trichoderma atroviride SC1*.

Method of inoculation	***A. gallica* infected plants (%)**	
	1 year after inoculation	**2 years after inoculation**
Root Armillaria+bark Armillaria	80	90
Root Armillaria+sterile bark	60	60
Root Armillaria	70	70
Bark Armillaria	80	90
Sterile barks control	0	0
Untreated control	0	0

Table 1. Percentage of infected blueberry plants inoculated with coniferous bark (bark Armillaria) and/or infected wood pieces inserted between roots (root Armillaria), using an *A. gallica* isolate.

On grapevine and apple the application of the *T. atroviride* with shrimp shells at the time of re-planting was insufficient to reduce *A. mellea* and *R. necatrix* infection, respectively. When the application was carried out at least one month before re-planting the disease was better controlled. The application of *T. atroviride* with shrimps shells resulted in a reduction of *R.*

solani disease on potatoes under experimental semi-field application, but to a lesser extent under real field conditions. The difference was due to the lower concentration assessed by microbiological and molecular methods of *T. atroviride* in the soil found under field conditions.

The main recommendations and conclusions were:

- Different organic carriers, available at low cost as by-products of the wood industry or tannin extraction or fish farms, were evaluated.
    - Wood chips resulting from industrial tannin extraction did not increase the survival of Trichodermas even if infiltrated with nutritional components (yeast and malt extracts).
    - Trichoderma survival on bark and shrimp shells substrates was very high. Although there are differences between strains of Trichoderma in terms of survival and colonization, there were no difference in the impact of the different carriers on the strains, therefore a specific strain was selected as reference (T. *atroviride* SC1).
- Pre-treatment with bark mixtures (fir, larch and pine) or a by-product of shrimp farms (shrimps shells) increased the survival of Trichoderma atroviride SC1 (use as a reference strain) in soil.
- The inoculation of barks either reduced the risk of contamination with Armillaria mellea (when barks were infected by this pathogen) or reduced the incidence of the diseases when the soil was already infected.

3.3 To test the effect of *T. achaeae* rearing system on its efficacy as a BCA against tomato leafminer, *T. absoluta*.

Since arrival of the invasive pest tomato leafminer, *Tuta absoluta*, in Europe, different local natural enemies have been recorded on it prompting studies concerning their biology and performance as biocontrol agents. An exhaustive list of such insects which are able to attack *T. absoluta* is summarized by Desneux et al. (2010). Among these, one of the most promising is the egg parasitoid *Trichogramma achaeae* Nagaraja & Nagarkatti (Polaszek et al. 2012; Urbaneja et al. 2012) which was shown to be highly efficient in lowering infestation levels in experimental and commercial tomato greenhouses in southern Spain (Cabello et al. 2009; Desneux et al. 2010; Chailleux et al. 2012). Based on these observations, different companies have started commercial production and distribution of *Trichogramma*-parasitized eggs of *Ephestia kuehniella* provided as user-friendly dispensers, glued on cardboard. The strategy suggested for its use in protected tomato crops is inundative release, as already used in other biological control programmes deploying Trichogrammatidae as BCAs (Consoli et al. 2010). Following producer and researchers' advice, *T. achaeae*, should be released at a rate of 25-50 adults m^{-2} twice a week, according to the level of pest infestation.

The rearing system (plant + host eggs on which the insect reared by the producer) can have a substantial impact on the fitness of egg parasitoids and hence their performance as BCAs. For example, *Trichogramma* strains collected from Noctuidae, Plutellidae and Crambidae on Solanaceae, Oleaceae and Vitaceae effectively parasitized *T. absoluta* eggs (Chailleux et al. 2012). Similarly, the nutritional quality of the prey/host can affect BCA performance (review in Toft 2013; Jensen et al. 2012; Mayntz et al. 2005).

Two main approaches are available to enhance the parasitoid's preference for the target host: 1. selective rearing on the target host and 2. priming with the target host before release. The best known successful case is the mass rearing of *Trichogramma brassica* on *Ephehestia kuhniella* (flour moth) for rapid augmentation of population numbers, combined with rearing for one generation on the target host, the European corn borer, *Ostrinia nubilalis*

(Sigsgaard 2005). This approach provides both the benefit of mass rearing and suitable host specialisation.

In general we found better performance of *Trichogramma achaeae* reared on *Tuta absoluta* eggs in terms of longevity when compared to those reared on *Sitotroga cerealella* (A06) or *Ephestia kuehniella* (A02) eggs. Rearing *T. achaeae* on *T. absoluta* eggs (TaA02 and TaA06) resulted in an almost double longevity in respect to that of parasitoids reared on *E. kuehniella* eggs and *S. cerealella* (One Way Anova, P < 0.001.) (Fig. 1). As expected, we recorded an overall higher longevity for females as compared to males (One Way ANOVA, P < 0.001) and for parasitoids fed with honey compared to unfed ones (Two Way ANOVA, P < 0.001). No statistical differences were noted between the longevity of males and females from A02 and A06 strains.

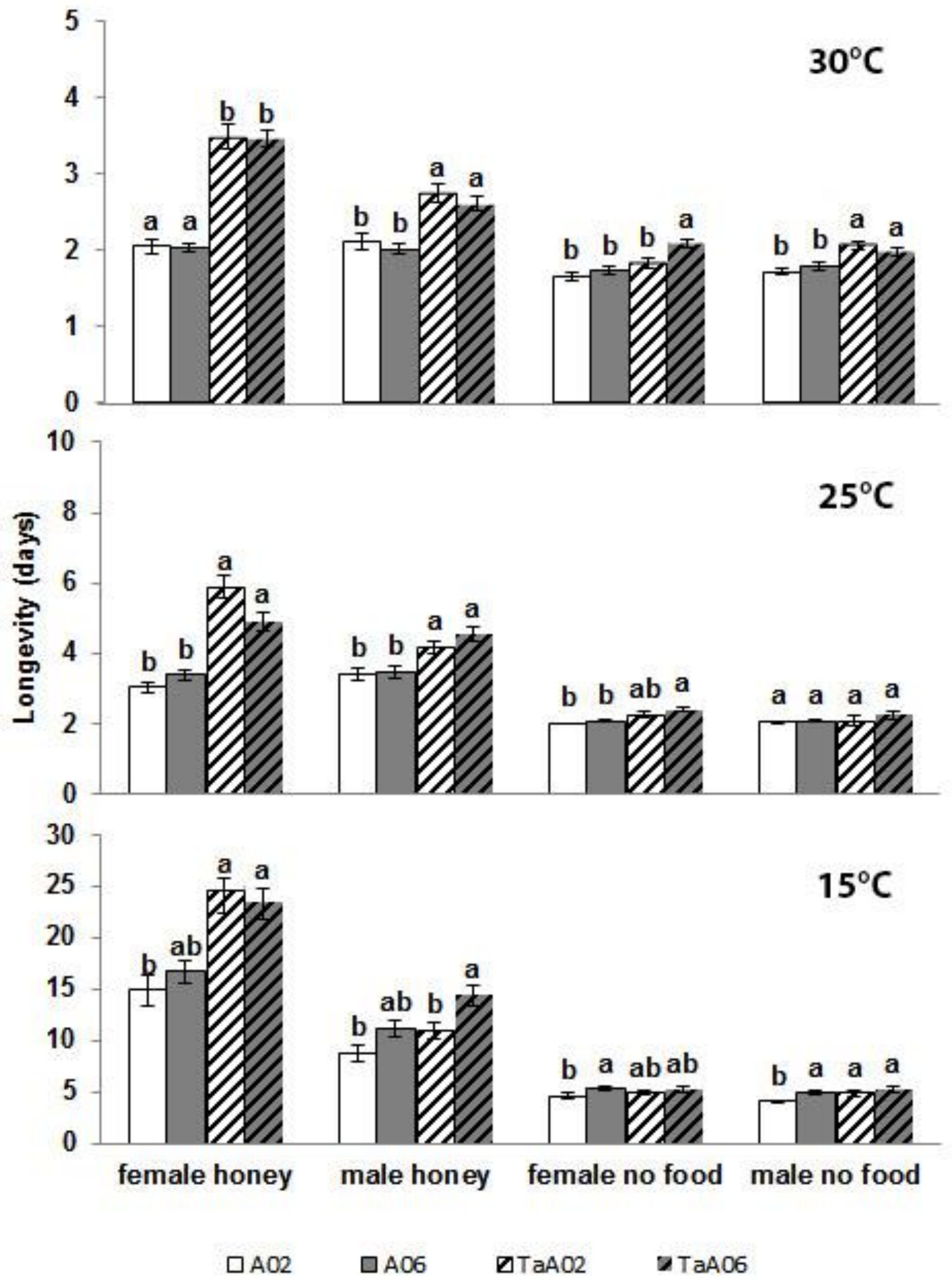

Fig 1. Effect of rearing system on survival of *Trichogramma achaeae*. Females and males were provided either with honey or no food. A02 insects were reared on *Ephestia kuehniella* eggs, A06 insects were reared on *Sitotroga cerealella* eggs. TaA02 and Ta06 insects were reared on *Tuta absoluta* eggs (after having been taken from cultures reared on *Ephestia kuehniella* and *Sitotroga cerealella* for TaA02 and TaA06 respectively).

Fertility of *T. achaeae* was similarly affected by the rearing system. In general, we found that parasitoids performed better when the rearing system corresponded to the target one. The highest parasitism rate was recorded for parasitoids reared on and offered *E. kuehniella* eggs (Fig. 2). The lowest parasitism rate was recorded for parasitoids reared on *E. kuehniella* eggs and offered *T. absoluta* eggs. However, a single generation on *T. absoluta* improved the performance of parasitoids coming from *E. kuehniella* or *S. cerealella* when they were offered *T. absoluta* eggs (Figure 2).

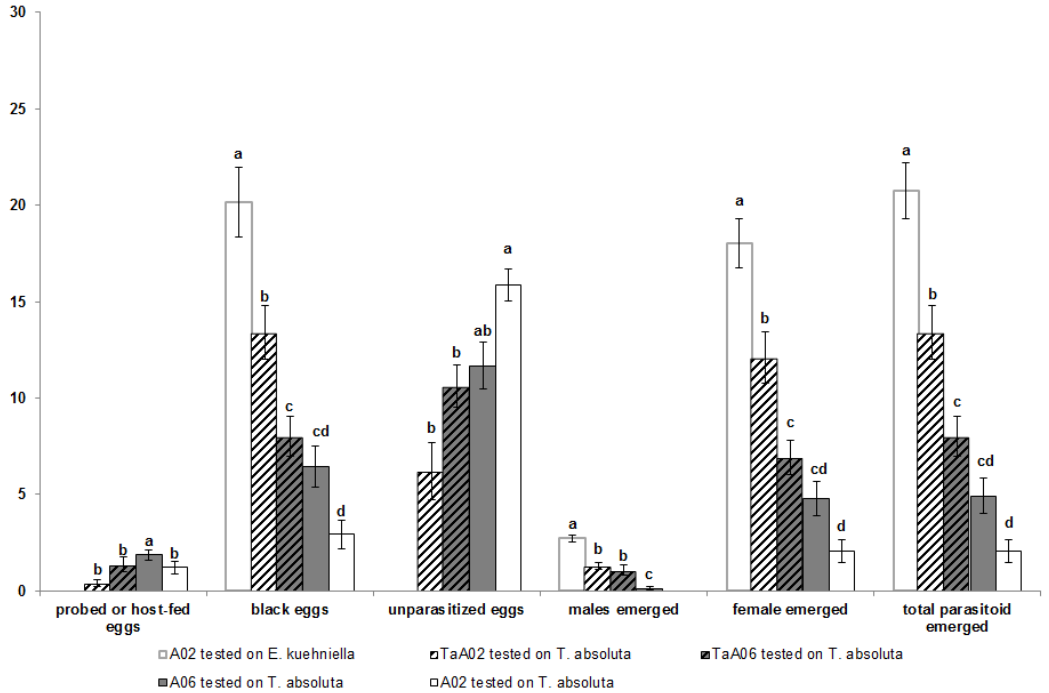

Fig 2. Effect of rearing system on fecundity of *Trichogramma achaeae* A02 insects were reared on *Ephestia kuehniella* eggs, A06 insects were reared on *Sitotroga cerealella* eggs. TaA02 and Ta06 insects were reared on *Tuta absoluta* eggs (after having been taken from cultures reared on *Ephestia kuehniella* and *Sitotroga cerealella* for TaA02 and TaA06 respectively).

3.4 To test if preimaginal thermal acclimation influences parasitism rate of *T. achaeae* on *T. absoluta*.

Along with rearing system, temperature during development and release can have a marked effect on the performance of BCAs and field performance can be improved through acclimation to a relevant temperature. Thermal conditions during the development of immature stages, especially idiobionts such as *Trichogrammatidae*, has considerable impact on adult fitness (Hoffmann and Hewa-Kapuge 2000; Lessard and Boivin 2013). For example, acclimation at 13°C of preimaginal *T. brassicae* stages improves the fitness of the resulting adults, particularly in terms of longevity and fecundity (Lessard and Boivin 2013). This can be used as a valuable tool for optimizing *T. achaeae* performance in different thermal environments. To explore the potential of this approach, we combined three different rearing systems (*Ephestia kuehniella* eggs + no plant, *Sitotroga cerealella* eggs + no plant and *Tuta absoluta* eggs + tomato plant) and three acclimation temperatures (15°C, 25°C, 35°C), assessing the resulting fitness and parasitism rate at five different temperatures (10°C, 15°C, 25°C, 30°C, 35°).

We found that *T. achaeae* performed best when the developmental acclimation temperature matched the one at oviposition (Fig. 3 A, B). For example, when offered host eggs at low temperature, females acclimated at 15 °C parasitized more eggs than females acclimated at higher temperature (Fig 3; $P < 0.001$). Likewise, females acclimated at 30 °C parasitized more eggs than females acclimated at low temperature, when exposed to high temperatures. At 35 °C there was no difference in the mean number of black eggs per exposed female (Fig. 3B), even though we recorded a higher percentage of parasitizing females acclimated at 30°C as compared to those acclimated at 15°C and 25°C (Fig. 3A, $P < 0.01$). All these differences vanished at 25°C (Fig. 3).

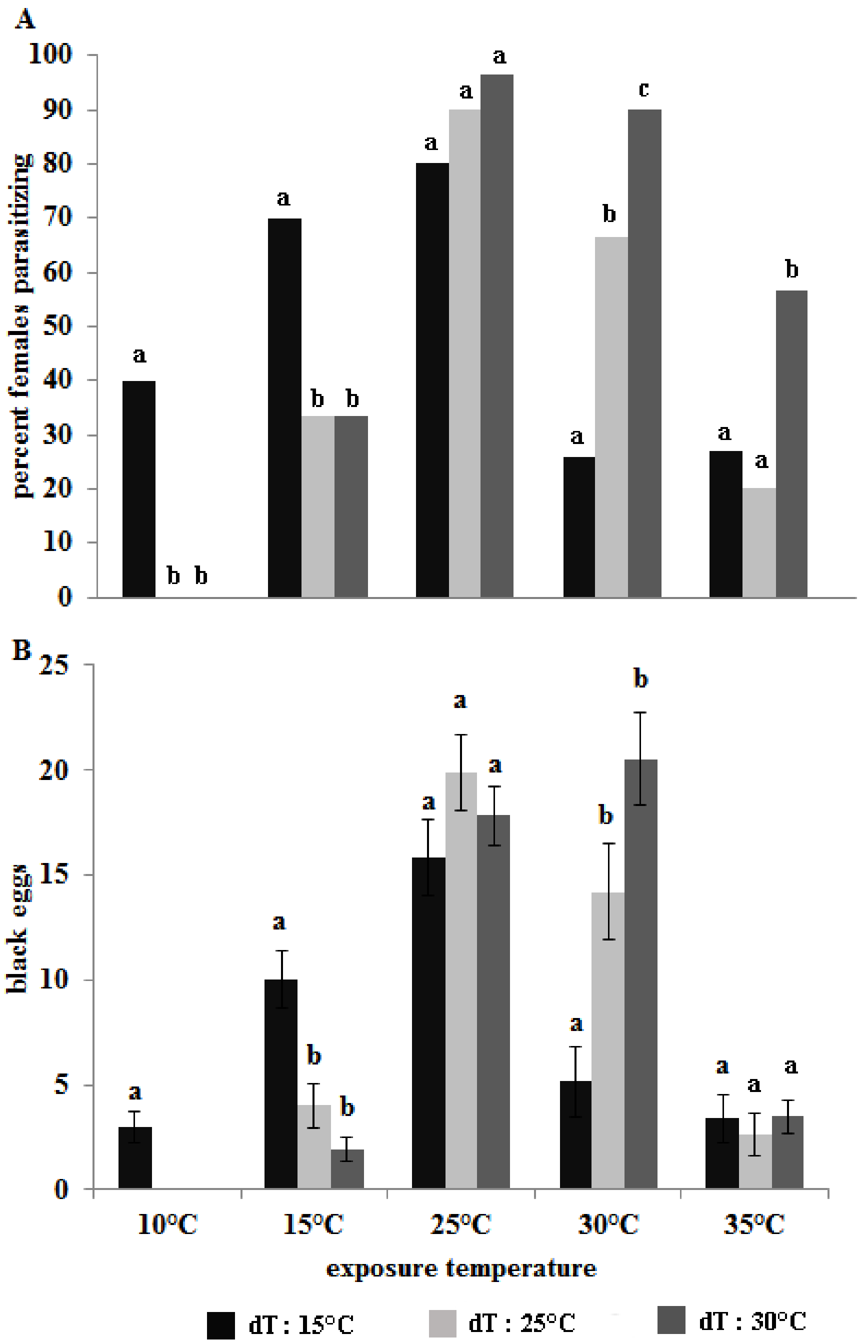

Fig 2. Effect of developmental temperature (dT) on *Trichogramma achaeae* fertility

Even though parasitism rates may reach about 30% (Chailleux et al. 2012; Do Thi Khanh et al. 2012) *T. achaeae* mass releases have not on their own been considered sufficient and a combination with other biological control methods (mirid predators and mating disruption) is recommended (Desneux et al. 2010). *T. achaeae* should be released at the onset of tomato-growing cycle, when other antagonists of *T. absoluta* are not yet well established in tomato fields. For example, at low densities of *T. absoluta*, myrid predators can behave as phytophagous insects. The low parasitism rate, lack of diapause during cold storage and absence of thelytoky with *T. achaeae* have encouraged studies to find less expensive and more efficient *Trichogamma* species (Chailleux et al. 2012; Do Thi Khanh et al. 2012). However, an alternative to *T. achaeae* has not yet been found and this wasp remains the best parasitoid species to be used in biological control programmes against the tomato borer.

4. Conclusion

A. quisqualis should be used under suitable environmental conditions. If it is targeted against overwintering fruiting bodies of powdery mildews the treatment should be applied when the fruiting bodies are not completely mature (white and yellow stage). A water extract of shrimp shells can be used for a pre-germination treatment of *A. quisqualis* conidia in order to speed up germination. This results in an increased efficacy against powdery mildews of cucurbits, strawberry, grapevine and apple in commercial-like conditions. This effect is due to the reduced time of exposure to adverse environmental conditions between treatment and penetration of the powdery mildew host by *A. quisqualis.*

The use of suitable carriers (i.e. barks, shrimp shells) can increase the survival and efficacy of Trichodermas against soil-borne disease, however enough time should be given to the biocontrol agent in order to reduce the inoculum of the pathogen before replanting the new vineyard/orchard.

We found it was possible to improve the fitness and the parasitization efficiency of *T. achaeae* on *T. absoluta* eggs through a combination of rearing system and temperature treatments. For better performance, at least one generation of the parasitoid should be completed on the same host before its release. Furthermore, BCA performance and efficacy against the target pest is maximised when rearing temperatures are the similar to those experienced in the field into which they are delivered. This result demonstrates the importance of acclimation of the parasitoids at the same temperatures that are likely to be found in the field in which they are released, according to season and cultural conditions (greenhouse, open field) to achieve a better control of *T. absoluta* with *T. achaeae*.

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