Short communication

New approaches for the sustainable management of *Neopestalotiopsis clavispora*, the causal agent of strawberry crown and root rot in Tucumán, Argentina

Nuevos enfoques para el manejo sustentable de *Neopestalotiopsis clavispora*, agente causal de la Podredumbre de raíces y corona de la frutilla en Tucumán, Argentina

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Abstract

The disease caused by *Neopestalotiopsis clavispora* in strawberries is responsible for severe damage and is considered an emerging threat worldwide. It was first identified as causing significant losses in commercial fields in Argentina in 2016. This study aimed to evaluate the efficacy of various biological control agents for managing *N. clavispora* within a sustainable strawberry crop management framework. The effectiveness of *Bacillus methylotrophicus*, *Azospirillum argentinense* REC3, two strains of *Trichoderma*, and a low-toxicity inorganic compound against the pathogen was assessed through *in vitro* and *in vivo* assays. Additionally, the resistance of 11 commercial strawberry cultivars to the pathogen was investigated using artificial inoculations. The results showed that, *in vitro*, both *Trichoderma* strains exhibited higher inhibition of *N. clavispora* (87-90%) compared to *B. methylotrophicus* (61%). Regarding cultivar performance, significant differences in disease severity were observed after artificial inoculations on detached leaves. The cultivars Sabrina, Sayulita, San Andreas, and Sahara exhibited foliar damage levels below 20%. Preventive applications of *Trichoderma* improved root length, and fresh and dry root weight, with mild symptoms similar to that of the fungicide Bellis® used as a chemical control. Applications of *A. argentinense* REC3 significantly reduced disease severity with and also enhanced leaf biomass, increasing aerial fresh and dry weight as well as the leaf area index. These findings provide valuable insights for developing integrated management strategies for this disease, ensuring environmental sustainability, maintaining fruit quality, and preserving biodiversity in strawberry production agroecosystems.

Keywords: *Neopestalotiopsis;* Plant disease management; Plant rot; Strawberry.

Resumen

La enfermedad causada por *Neopestalotiopsis clavispora* en frutilla es responsable de severos daños y es considerada una enfermedad emergente a nivel mundial. Fue identificada como causante de graves pérdidas en campos comerciales en Argentina en 2016. Este trabajo tuvo como objetivo evaluar diferentes alternativas biológicas para el control de *N. clavispora* dentro de un esquema de manejo sustentable para el cultivo de frutilla. A través de ensayos, *in vitro* e *in vivo*, se evaluó la eficacia comparativa del control de *Bacillus methylotrophicus*, *Azospirillum argentinense* REC3, dos cepas de *Trichoderma* y un compuesto inorgánico de baja toxicidad frente al patógeno. También se estudió el comportamiento sanitario de 11 cultivares comerciales de frutilla frente al patógeno mediante inoculaciones artificiales. Los resultados indicaron que ambas cepas de *Trichoderma* mostraron *in vitro* mayores porcentajes de inhibición de *N. clavispora* (87-90%) en comparación con *B. methylotrophicus* (61%). En cuanto al comportamiento de los cultivares después de inoculaciones artificiales sobre hojas desprendidas, se observaron diferencias significativas. Los cultivares Sabrina, Sayulita, San Andreas and Sahara presentaron menor superficie foliar dañada, por debajo del 20%. Finalmente, las aplicaciones preventivas con *Trichoderma* incrementaron la longitud radicular, el peso fresco y seco radicular, y presentaron valores de severidad similares a las tratadas con el fungicida Bellis®, usado como control químico. Las aplicaciones de *A. argentinense* REC 3 redujeron significativamente la severidad de la enfermedad y además estimularon la biomasa foliar, incrementando el peso fresco y seco de los tejidos aéreos y el índice de área foliar. Los resultados aportan información relevante para establecer medidas eficientes para el manejo integrado de esta enfermedad, inocuas para el medio ambiente, cuidando la calidad de la fruta y preservando la biodiversidad en el agroecosistema de las zonas de producción de frutilla.

Palabras clave: Frutilla; Manejo de enfermedades de plantas; *Neopestalotiopsis*; Podredumbre de plantas.

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In Argentina, around 2000 ha of strawberries (*Fragaria* × *ananassa* Duch.) are cultivated, with an average annual production of 70,000 t (Maza *et al.*, 2024). Buenos Aires, Tucumán, and Santa Fe provinces account for about 70% of the country's total production (Kirschbaum *et al.*, 2017). In strawberries, yield losses systematically occur due to different causes, with fungal diseases being the most frequent. Since 2016, severe damage has been observed in commercial strawberry fields, associated with the fungus *Neopestalotiopsis clavispora*, this being its first identification in Argentina (Obregón *et al.*, 2018). *N. clavispora* presents white–yellow colonies with wavy edges and whitish aerial mycelium, and an optimal growth rate at 28 °C. It produces black, acervulate-type fructifications or bodies, with fusoid or ellipsoid conidia with five cells. The apical cell has several appendages, the basal one a single appendage, and three consecutive middle cells, colored in different dark tones (Maharachchikumbura *et al.*, 2014).

Among the symptoms induced by this pathogen, reddish-brown interveinal spots are described on young leaves, and rust-colored areas -that start from the margins and expand to cover the entire leaflet as the disease develops- in mature leaves. These symptoms are consistent with necrotic areas and discoloration in the root and crown, which can ultimately cause the death of the plant. Currently, *N. clavispora* is cited as a pathogen that causes severe damage to strawberry crops and is considered worldwide as an “emerging disease” (Baggio *et al.*, 2021). It was first reported in Spain as the causal agent of root and crown rot in strawberries (Chamorro *et al.*, 2016), and was simultaneously detected in China (Zhao *et al.*, 2016). Other authors link *N. clavispora* as a causal agent of fruit rot, recognizing several *Neopestalotiopsis* species associated with the damage (Ayoubi and Soleimani, 2016; Baggio *et al.*, 2021). In Argentina, its presence was confirmed in the provinces of Tucumán (Famaillá) and Corrientes (Bella Vista), causing severe damage to commercial strawberry fields (Obregón *et al.*, 2018). The conventional method for controlling pathogenic fungi typically involves the application of fungicides. However, chemical pesticides can raise significant problems, such as absorption and accumulation in fruit, as well as disruption of environmental balance. The use of biological control agents, such as antagonistic microbes (e.g., *Trichoderma* spp. and *Bacillus* spp.) that reside in plant tissues (endophytes) and roots, represents an environmentally sustainable alternative for managing certain diseases. Pre-screening of strawberry cultivars for disease resistance prior to their introduction and commercial use is also crucial for effective disease management. Recently, both biological and chemical fungicides have been evaluated for their preventive and curative efficacy in controlling *Neopestalotiopsis rosae* in strawberries under greenhouse conditions. These studies demonstrated that certain treatments were more effective when applied preventively (Acosta-González *et al.*, 2024). Furthermore, it has been reported that strawberry cultivars exhibit varying levels of resistance to crown rot disease, with significant differences in disease intensity observed among the three cultivars. Additionally, the application of a bacterial consortium and *T. harzianum*, combined with artificial inoculation of the pathogen via drenching, demonstrated variable effects on plant growth depending on the growth medium (Widyaningsih and Triasih, 2021). This work aimed to evaluate the i*n vitro* and *in vivo* efficacy of different strategies for the integrated management of strawberry crown and root rot diseases. *In vitro* assays were conducted to test antagonistic microorganisms that inhibit the pathogen growth directly. The *Bacillus* group is known for producing a variety of bioactive metabolites that lead to antibiosis and enable competition for space and nutrients. The effectiveness of *B. subtilis* and other species in inhibiting the reproduction of pathogenic fungi to control diseases in various plants has shown significant results (Chen *et al.*, 2009). *Trichoderma* spp. utilizes multiple antagonistic mechanisms against crop pathogens, such as lytic enzymes, mycoparasitism, and competition for nutrients and space (Mukherjee *et al.*, 2012). For the *in vivo* trials, the strain REC3 of *A. argentinense* was also included in the preventive applications due to its reported efficacy as a plant growth-promoting bacterium in strawberry fields (Pedraza *et al.*, 2007), as well as its ability to activate plant defense mechanisms, leading to systemic resistance against pathogen infections. Recently, REC3 was found to reduce plant mortality through root inoculations or foliar treatments with its protein AzFlap, demonstrating the effectiveness of both treatments in controlling *M. phaseolina* in strawberry plants (Elias *et al.*, 2021). The first assays involved evaluating the *in vitro* control of *Bacillus methylotrophicus* and two strains of *Trichoderma* (*T.* INTA and *T. asperellum*) to inhibit the growth of the pathogen using dual antagonistic cultures. A 5 mm Ø disc of actively growing pathogen mycelium was placed on *Petri* plates containing potato dextrose agar (PDA) and incubated for 48 hours in the dark at 25 ± 1 °C. Subsequently, a streak or a 5 mm Ø disc of the biocontroller was placed 3 cm apart from the pathogen disc. The plates were incubated for 7 days. The antagonistic action was calculated using the percentage of growth inhibition (PGI), determined by the growth area of the phytopathogen in the presence of the antagonist (C1) compared to the development of the pure culture (C2), where PGI = [(C1 - C2) / C1] x 100. For *Trichoderma* spp., the contact time (CT), which is the number of days in which the antagonist and the pathogen come into contact, was also measured. Finally both strains were classified using a qualitative scale of five degrees (Bell *et al*., 1982). Three replicates of each condition were performed, and the assays were repeated three times. Data were analyzed using ANOVA followed by Fisher's least significant difference (LSD) calculated at *P* = 0.05. In the second trial, the behavior of 11 strawberry cultivars against the disease was evaluated using detached leaves challenged with inoculation of *N. clavispora,* under controlled conditions. Two asymptomatic leaves were taken from the cultivars Benicia, Camino Real, Fronteras, Petaluma, Rábida, Rociera, Sabrina, Sahara, San Andreas, Savana, and Sayulita, and placed in humid chambers, at 95% relative humidity. A 5 mm Ø mycelium disk of the pathogen, cultivated for 7 days on PDA, was placed on the upper surface of each leaflet. Sterile PDA disks were used as controls. The humid chambers were incubated in a growth room at 28 ± 2 °C with a 16-8 hour photoperiod, for 10 days. Cultivar performance was quantitatively assessed by symptom severity. The estimation of severity was calculated from the surface area of the damaged leaf relative to the total leaf area, expressed as a percentage using the software ImageJ (Rasband, 2018). Based on these values, an arbitrary scale was established to classify the different cultivars as tolerant (<20% damaged area), moderately susceptible (20–50% damaged area), or susceptible (>50% damaged area). The statistical analysis was over 9 leaflets per cultivar, and this assay was conducted twice. Data were analyzed by ANOVA, followed by Fisher's least significant difference (LSD) test at *P* = 0.05. The third trial aimed to compare the *in vivo* performance of preventive applications of *Azospirillum argentinense* REC3, *Bacillus methylotrophicus, Trichoderma* INTA, *T. asperellum*, and chlorine dioxide (ClO_2), using Bellis® fungicide and water as chemical and absolute controls, respectively. In this trial 6 replicates of Benicia plants in plastic pots were grown under greenhouse conditions. Twenty-four hours after the applications, the plants were artificially inoculated by placing two discs of *N. clavispora* pure culture on the crown, which had been previously wounded with a sterile scalpel. After 21 days post-inoculation, the plants were uprooted, and the intensity of the disease and other phenotypic parameters such as fresh and dry weight of the aerial part (AFW and ADW) and root part (RFW and RDW), leaf area index (LAI), and root length (RL) were measured. The effectiveness of preventive treatments was estimated by evaluating the intensity of disease symptoms. The intensity value was calculated by scoring crown rot symptoms on strawberry plants using a scale from 0 to 4, where: 0 = healthy plants, 1 = >10% mild pathogen attack, 2 = 11-25% moderate attack, 3 = 26-50% severe attack, and 4 = >51% plant death (Dwiastuti *et al*., 2015). The results indicated *in vitro* average PGI values of 61.1% for *B. methylotrophicus*; while the *Trichoderma* strains presented higher percentages of inhibition with PGIs of 89.7 and 86.8% for *T. asperellum* and *T.* INTA, respectively (Figure 1a). The Bell scale indicated that both *Trichoderma* strains were highly antagonistic (Classes 1 and 2). Finally, *T. asperellum* presented a TC of 60 h, while *T.* INTA 48 h. Regarding the performance of the cultivars, the evaluation showed all were susceptible to the disease through artificial inoculations. Although the LSD Fisher Test, showed significant statistical differential cultivar behavior (p-Value =0.10, DMS=7.01859, standar error= 2.76). Damage mean values for Sabrina 5.12%, Sayulita 13.36%, San Andreas 17.41% and Sahara 17.53% reached the highest tolerance, with severity values below 20%; cultivars Savana and Camino Real in a moderate susceptibility group with severity values of 25.87 and 37.01% , respectively; finally Fronteras 53.62%, Petaluma 54.28%, Benicia 62.38%, Rabida 63.2% and Rociera 75.25%, scored them as more susceptible cultivars with percentages over 50% of foliar damage (Figure 1b y c). Finally, all preventive applications of biological agents in strawberry plants reduced the intensity of symptoms caused by *N. clavispora*. Additionally, *Trichoderma* treatments increased RL, RFW and RDW, showing a similar behavior to the chemical control. Applications of *A. argentinense* REC 3 significantly stimulated foliar biomass, AFW, ADW, and LAI. Chlorine dioxide did not differ from the absolute control, and *B. methylotrophicus* exhibited erratic behavior. The mean outputs of the LSD Fisher statistical test for mean comparison, with a significance level of 1% and standar error (E.E) for each evaluated parameter are presented in Table 1. All these results suggest that various effective management tools can be integrated to combat this disease. Cultivar selection and preventive applications with biological agents are strategies that could reduce *Neopestalotiopsis clavispora* damage, ensuring the quality and safety of the fruit and preserving biodiversity in the agroecosystem of strawberry production areas. Future studies should be conducted to further investigate the results observed in this study, as this is intended to be a short communication.

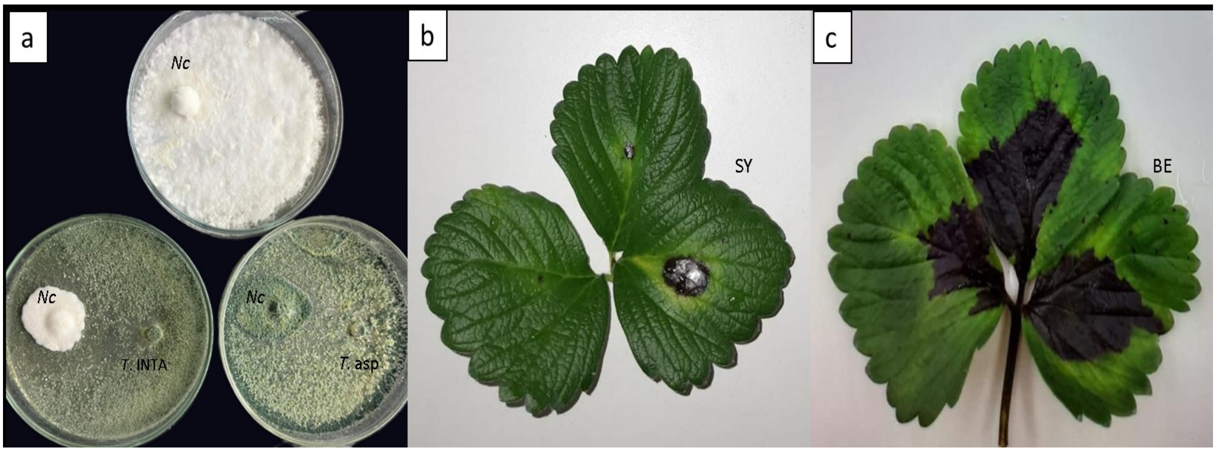

Figure 1. Use of biological fungicides and cultivar behavior as effective tools for the sustainable management of *Neopestalotiosis clavispora*. **a**, *In vitro* antagonism of *Trichoderma* INTA (T. INTA) and *T. asperellum* (T. asp) showing severe growth inhibition of *N. clavispora* (Nc). **b**, Foliar disease symptoms induced on detached leaf of cultivar Sayulita (SY) and **c**, cultivar Benicia (BE) 7 days post artifical inoculation, incubated at 28°C.

Table 1: *In vivo* assays. Effects of preventive treatments on strawberries plants cv Benicia, 21-days post inoculation with *Neopestalotiopsis clavispora*.

Treatments	Mean comparison of parameters evaluated						
	DI	RL (cm)	RDW (g)	RFW (g)	ADW (g)	AFW (g)	LAI
Absolut control	4	19.7±2.58a	3.88 ±0.51a	7.79±0.8a	3.21±0.24abc	12.92± 0.31a	4.79±1.27a
Bellis©	1	22.1±2.12ab	5.3±1.3ab	11.93±1.82c	3.27±0.67abc	13.43±2.23a	6.87±3.82a
ClO_2	4	16.83±6.62a	3.92±1.45a	8.65±2.70ab	2.57±1.32a	13.01±3.89a	2.54±2.89a
T. asperellum	1	26.2±5.59b	6.16±0.92b	11.06±1.49bc	2.97±0.42ab	15.34± 1.21ab	34.06±8.29ab
T. INTA	1	18.5±4.69a	5.3±0.73ab	9.98±1.45abc	2.48±0.64a	13.14 ±1.58a	35.48± 5.30ab
B. methylotrophycus	2/3	16.95±1.97a	4.62±1.08ab	9.87±2.29abc	4.01±0.72bc	18.85±2.98bc	79.83± 62.13bc
A. argentinense REC 3	1	16.9±3.15a	5.25±0.75ab	11.4±1.37c	4.27±1.08c	20.24±4.69c	119.74±115.66bc

Mean value ±standard deviatiaton. DI: disease intensity from 0 to 4, where: 0 = healthy plants, 1 = >10% mild pathogen attack, 2 = 11-25% moderate attack, 3 = 26-50% severe attack, and 4 = >51% plant death.. RL:root lenght, RDW: root dry weight, RFW: root fresh weight, ADW: aerial dry weight, AFW:aerial fresh weight, LAI: leaf area index. Test:LSD Fisher, P=0,01. Different letters in each column indicate significant statistical differences between treatments.

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