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Copper-alternative products to control anthracnose and Alternaria Brown spot on fruit of Tarocco sweet oranges and lemon in Italy

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ABSTRACT

Colletotrichum and *Alternaria* species, causal agents of pre- and postharvest anthracnose and Alternaria brown spot (ABS) on fruit respectively, have been recently reported as emerging fungal pathogens on citrus in the Mediterranean area, causing severe economic losses. The control of these pathogens is increasingly problematic, particularly in organic citrus orchards where disease management largely relies on the use of copper-based antimicrobials. With limitations in the use of Cu compounds imposed by the European Commission, due to the demonstrated noxious effects on environment, research for alternative formulations is encouraged. In this work, copper-alternative products (basic substances, plant extract, biocontrol agent and their combinations) were tested on sweet orange clones ('Tarocco Scirè' and 'Tarocco Tapi') and lemon clone ('Femminello Siracusano 2 KR') for two - three consecutive growing seasons in three citrus orchards within one of the most representative Italian citrus production areas to control natural infections on fruit caused by *Colletotrichum* and *Alternaria* species. Results showed that, even under different disease pressure levels, chitosan, *Equisetum arvense* and sweet orange essential oil-based products, alone and in mixture, significantly reduced disease incidence and severity compared with untreated controls, often showing comparable or better efficacy than copper. The copper-alternative products were also subjected to benefit-cost analysis, that showed an increase in the costs of phytosanitary treatments, differing according to the products, often covered by a positive increase in the marketable production compared to untreated control or copper-treated fruits. The good efficacy of copper-alternative products, alone and in mixture, indicates the potential of their sustainable and large-scale use, useful for replacing or reducing the use of copper in integrated and organic citriculture.

1. Introduction

The cultivation of citrus represents one of the most important industries worldwide, with a continuously increasing in production trades during recent decades. Several countries of Mediterranean basin such as Greece, Italy, Spain, Tunisia and Turkey are important producers of citrus fruit, as well as additional regions characterized by a Mediterranean climate, such as Australia, California, Florida and South Africa (FAOSTAT, 2022). Among them, Italy is the second citrus producer in Europe, with a production of 3 million tonnes during 2022 (Eurostat, 2023). Additionally, Italy is the leading producer country for organic citrus farming globally, with over 31.000 ha of organic citrus area dedicated (Sinab.it). Due to favourable soil and climate conditions, a large part of Italian citrus production is localized in Sicily, accounting for approximately 58% and 87% of the total Italian production of sweet oranges and lemon respectively (Istat.it), showing a wide panorama of varieties cultivated in the region. The best quality of the Sicilian citrus supply is represented by the production of pigmented ("blood") oranges. Among all the Italian blood oranges, 'Tarocco', with its several clones, is the most widely cultivated cultivar in eastern Sicily, in the area between the provinces of Catania, Enna and Syracuse, in the south and south-west of Mount Etna, and recognized as Protected Geographical Indication (PGI) "Arancia Rossa di Sicilia" by the European Union (Commission Regulation No 1107/96 of 12 June 1996). As well as the lemon cultivation encompasses several cultivars of great economic interest in the Sicilian areas, where pedological and weather conditions allow to reach high quality standards. In particular, the lemon 'Femminello Siracusano', appreciated for its unique aroma, high acidity and extraordinary richness of juice, has obtained the PGI recognition with the denomination "Limone di Siracusa" (Commission Regulation No 96/2011 of 3 February 2011).

Unfortunately, a broad range of fungal species causes diseases

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affecting roots, foliage, fruit and wood where *Citrus* spp. are cultivated. Among them, *Colletotrichum* and *Alternaria* species have been in the last decade reported as emerging fungal pathogens on citrus on Mediterranean basin, including Sicilian areas (Aiello et al., 2015, 2020; Uysal et al., 2022). Anthracnose of citrus fruits caused by *Colletotrichum* spp. is a severe disease (Guarnaccia et al., 2017) and the main causal agents *C. gloeosporioides* (Penz.) Penz. & Sacc. have been treated for decades as postharvest pathogens. Only recently *C. gloeosporioides* and *C. karsti* You L., Yang, Zuo Y. Lim, K.D. Hyde & L. Cai (as '*karstii*') (Yang et al., 2011) have been also associated to a broad variety of preharvest symptoms, including heavy anthracnose on leaves and fruit, fruit drop, twig dieback and defoliation on several citrus accessions, including 'Tarocco' group (Aiello et al., 2015; Vitale et al., 2021), heavily compromising the production. Alternaria brown spot (ABS) is a serious postharvest disease of citrus in highly susceptible cultivars (Garganese et al., 2016; Peever et al., 2004; Timmer et al., 2003). It has been recently described as responsible for depressed brown to black lesions on ripe and unripe fruits, also surrounded by a yellow halo, in several citrus accessions cultivated in Italy (Aiello et al., 2020; Vitale et al., 2021), causing significant losses of yield and marketable fruit. ABS is mainly caused by *A. alternata* (Fr.) Keissl., and occasionally *A. arborescens* E.G. Simmons has been recovered from symptomatic fruit (Aiello et al., 2020; Garganese et al., 2016). Moreover, a broad degree of susceptibility in the field was recently observed within citrus accessions cultivated in Sicily, clearly indicating an extreme degree of susceptibility of some 'Tarocco' accessions (e.g., 'Tarocco Sciara', 'Tarocco Scirè VCR' and 'Tarocco Emanuele') to preharvest anthracnose symptoms on fruit, and a higher susceptibility of lemon to ABS on fruits, being 'Femminello Siracusano 2 KR' the most susceptible lemon accession (Aiello et al., 2020; Vitale et al., 2021).

Currently, chemical management is the main strategy to control Colletotrichum and Alternaria diseases on citrus worldwide, and fludioxonil (with a temporary authorization, according to Dec. October 28, 2022, art. 53 CE Reg. 1107/2009) (Regulation No 1107/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 21 October 2009), pyraclostrobin and Cu compounds are the only registered fungicides approved in integrated citrus production in Italy (Fitogest, 2023a; Fitogest, 2023b). The active ingredients fludioxonil and pyraclostrobin have been suspected to lead harmful consequences for humans (Çayır et al., 2016; Lee et al., 2019). Furthermore, only Cu compounds are allowed in organic agriculture, but the use of metallic copper is currently limited to 28 kg/ha in 7 years (European Union. Regulation EU 2018/1981 of the European Parliament and of the Council of 13 December 2018, 2018) due to its toxicity to insects, plants, soil fauna and soil microbiota (Lamichhane et al., 2018). Moreover, the management of citrus anthracnose and ABS results particularly changeling in presence of favourable environmental conditions for the pathogens (Timmer et al., 1998; Silva et al., 2017; Riolo et al., 2021), and the number of needed Cu-treatments for the containment of the diseases may change according to the climatic conditions and susceptible host, and it may easily result in an overcome of recommended spray threshold (Katsoulas et al., 2020). Therefore, the demand for effective alternative substances less harmful for humans, animals and environment, that can replace copper compounds and traditional fungicidal active ingredients has increased. At the same time, to attain general acceptance by growers, the efficacy of alternative products should be at least comparable to the level of control provided by standard fungicides in natural disease conditions and should be validated in a wide range of scenarios (i.e., disease pressure, cultivars and accessions, agroclimatic conditions, cultivated areas).

Among alternative products, plant extracts, basic substances (BSs) and biological control agents (BCAs), included in Annex II of the Commission Regulation (EC) 889/2008 (Commission Regulation No 889/2008, 2008), which lists the products permitted for plant protection in organic crop production, are gaining attention for the management of fungal pathogens. Some alternative products based on BSs and plant extracts, already introduced in the marked as commercial formulations and authorized for their use on citrus crop, such as chitosan and sweet orange essential oil-based products, have been recently tested for the control of anthracnose caused by *Colletotrichum* spp. on sweet orange 'Tarocco Scirè' in open field, showing promising results (Lombardo et al., 2023). As well as for the commercially available alternative product based on *Equisetum arvense* L. (field horsetail), authorized for its use on citrus crop, has showed its inhibitory activity against *C. gloeosporioides* in laboratory screening in our previous study, and it deserves to be assessed in field conditions to evaluate the potential application as trade available formulation. Moreover, considering that an enhanced antifungal activity generally results from the formation of conjugate complexes between chitosan and other substances of natural origin (Buzón-Durán et al., 2019, 2020, 2021), and that legal framework would place no obstacle to a combined use of already approved basic substances, this possibility deserves to be explored too. Registered commercial formulation based on BCAs may be also used as a valid alternative to reduce copper applications (Dagostin et al., 2011), but only some of them are commonly used due to their variable effectiveness under commercial or open field conditions (Droby et al., 2016). Among them, a commercial product based on *Bacillus amyloliquefaciens* (former *subtilis*) strain QST 713 has been recently registered on its use on citrus crop. Although the potential of *Bacillus* species as biocontrol agents against citrus diseases has been widely highlighted (Chen et al., 2020), the effectiveness of *B. amyloliquefaciens* QST 713 in controlling preharvest ABS and anthracnose of citrus fruit have never been tested so far.

The promising results obtained in our previous work, together with the urge to find efficient copper-alternatives to control emerging citrus pathogens so to support Sicilian citriculture, has encouraged to extend the field trials in more citrus orchards under different conditions. Thus, in the present investigation, commercially available products based on basic substances (chitosan and *E. arvense*), plant extract (sweet orange essential oil), their mixtures and on BCA (*B. amyloliquefaciens* QST 713) were tested in pluriannual trials in three commercial citrus orchards located in representative Sicilian production areas against natural infections of anthracnose and ABS on fruit. Moreover, considering their presence in the market and their versatile applications, we hypothesize that the alternative products tested in this study could address a timely response to the urgent demand for products that can efficiently reduce the application of copper-based products in high-quality agricultural production.

Therefore, the main objectives of this study were: i) to confirm the previous study on the efficacy of selected alternative products and to evaluate their mixtures against anthracnose symptoms on fruit caused by *Colletotrichum* spp., broadening the study on differently susceptible 'Tarocco' blood orange accessions; ii) to validate, for the first time, their efficacy against ABS on fruit of lemon caused by *A. alternata*; iii) to evaluate the economic sustainability of their applications for a large-scale adoption; iv) to identify valuable and ready-to-use copper-alternatives for the sustainable control of emerging fungal citrus diseases.

2. Materials and methods

2.1. Study sites

A total of seven independent experimental field trials in three citrus orchards were carried out during two (2020 and 2021) and three (from 2019 to 2022) consecutive growing seasons to evaluate the efficacy of four alternative products and two mixtures against natural infections caused by *A. alternata*. and *Colletotrichum* spp. on lemon [*C. limon* (L.) Osbeck] and sweet orange [*C. sinensis* (L.) Osbeck]. The three citrus orchards were located in one of the most representative Italian citrus production areas in the eastern Sicily, falling within Syracuse and Catania provinces (Supplementary Fig. S1).

The three experimental orchards had a history of severe attacks of ABS on fruit on high susceptible lemon 'Femminello Siracusano 2 KR'

(Orchard I, Agnone) and of preharvest anthracnose on fruit on extremely susceptible orange 'Tarocco Scirè' (Orchard III, Pedagaggi) and on moderately susceptible orange 'Tarocco Tapi' (Orchard II, Mineo), widely documented in a previous work (Vitale et al., 2021). Thus, preharvest ABS symptoms on fruits of lemon 'Femminello Siracusano 2 KR' and anthracnose symptoms on fruit of oranges 'Tarocco' were always relatable to *Alternaria* and *Colletotrichum* species, respectively.

Specifically, the field trials were conducted in the following three orchards: Orchard I, located at Agnone, Augusta (37°18′26.0″N 15°05′09.1″E, altitude 2 m a.s.l., Syracuse province, Sicily, IT), on lemon 'Femminello Siracusano 2 KR' grafted onto Volkamerian lemon (*C. volkameriana* Ten. & Pasq.), during 2020 and 2021; Orchard II, located at Mineo (37°18′44.6″N 14°40′02.9″E, altitude 130 m a.s.l., Catania province, Sicily, IT), on sweet orange 'Tarocco Tapi' grafted onto sour orange (*C. aurantium* L.) during 2020 and 2021; Orchard III, located at Pedagaggi (37°11′ 30.27″ N, 14°55′ 37.52″ E, altitude 307 m a.s.l., Syracuse province, Sicily, IT), on sweet orange 'Tarocco Scirè' grafted onto sour orange during 2019/2020, 2020/2021 and 2021/2022 (Supplementary Table S1). Orchards I and III were managed following the integrated pest management principles, and Orchard II was managed according to organic farming regulations.

2.2. Climate data

The weather parameters, i.e., average temperature (°C), relative humidity (%) and rainfall (mm) were obtained from the data provided by the weather stations of Augusta (Syracuse province) for orchard I, Mineo (Catania province) for orchard II, and Francofonte (Syracuse province) for orchard III of Servizio Informativo Agrometeorologico Siciliano (SIAS), Sicily region. Data have been collected over the two and three-year period, encompassing the entire citrus fruit crop cycle.

2.3. Copper-alternative commercial products

The following alternative commercial products were tested in open field trials: (1) two basic substances [Chitosano Biorend® (Bioplanet; 1.9% chitosan hydrochloride; water-soluble liquid)] and Naturdai EQUIBASIC® (Idainature; 0.2% extract of *E. arvense*; water-soluble liquid)]; (2) one citrus essential oil [PREV-AM® PLUS (Ascenza; 5.8% sweet orange essential oil; water soluble liquid)]; (3) one biological control agent: [Serenade Aso® (Bayer; 14.1 g/L *Bacillus amyloliquefaciens* (former *subtilis*) QST 713)]. The following products were used in mixture: (4) Chitosano Biorend® + Prev-Am® Plus (Mixture 1); (5) Chitosano Biorend® + Naturdai Equibasic® (Mixture 2). Kop-Twin [Chimiberg-DIACHEM® (13.3% tribasic copper sulphate and 8.9% copper hydroxide)] was used as standard copper-based fungicide (Table 1).

2.4. Field application of alternative products

The trials included different number of field treatments at each orchard in each year, with four replicates of each treatment arranged in a completely randomized block design. Each replicate included four-six plants (depending on the number of fruits per plant). Buffer (untreated) plants were used to separate treated plots and replicates. Orchards were irrigated during summer and the fertilizers were distributed in the winter and spring, according to common practices for the areas. Each trial included an untreated control and a standard treatment of tribasic copper sulphate and copper hydroxide (Kop-Twin). In Orchard I, treatments were applied seven times during 2020 (21/07; 3/09; 17/09; 25/09; 5/10; 23/10; 6/11) and three times in 2021 (8/09; 24/09; 4/10); in Orchard II, treatments were applied five times during 2020 (28/05; 11/07; 13/08; 12/09; 14/10) and three times in 2021 (9/09; 27/09; 8/10); in Orchard III, treatments were applied three times during 2019/20 (27/12; 16/01; 1/02), four times in 2020/21 (15/12; 29/12; 20/01; 15/02) and three times in 2021/22 (7/12, 21/12; 20/01) (Table 1). The number of treatments varied depending on the meteorological events and the disease's symptoms monitoring. Treatments consisted in application of Chitosano Biorend (chitosan hydrochloride), Prev-Am Plus (sweet orange essential oil), Naturdai Equibasic (*E. arvense*), Mixture 1 [(Chitosano Biorend (chitosan hydrochloride) + Prev-Am Plus (sweet orange essential oil)], Mixture 2 [Chitosano Biorend (chitosan hydrochloride) + Naturdai Equibasic (*E. arvense*)] and Serenade Aso (*B. amyloliquefaciens* QST 713). Products were prepared following the label instructions and applied in the morning by a motorized backpack mist blower, approximately 2000 L/ha, and each plant was sprayed to runoff. The tested products, active ingredients, the application rates and the number of applications per trial are listed in Table 1.

2.5. Disease assessments

Disease assessments were aimed to evaluate the presence of preharvest symptoms of ABS and anthracnose on fruit, since in many citrus accessions severe infections compromise the marketability of fruits, thus causing heavy yield and economic losses. The disease monitoring (incidence and severity) was carried out in the field a variable number of times depending on the orchard and on the year of each trial. The last monitoring was always at harvest time. Specifically, disease incidence and severity were monitored three times from September through November in Orchard I and Orchard II during 2020 and 2021. In

Table 1
Treatments, products, application rates and frequencies used the in experimental orchards.

	Product trade name	Active ingredients	Supplier	Application rate	Number of field treatments						
					Orchard I		Orchard II		Orchard III		
					2020	2021	2020	2021	19/20	20/21	21/22
1	Chitosano Biorend®	Chitosan hydrochloride	Bioplanet S.r.l., Cesena, Italy	300 mL/hL	7	3	5	3	3	4	3
2	Naturdai EQUIBASIC®	*Equisetum arvense*	Idainature, Valencia, Spain	400 mL/hL	7	3	5	3	3	4	3
3	PREV-AM® PLUS	Sweet orange essential oil	Ascenza Italia S.r.l., Saronno, Italy	400 mL/hL	7	3	5	3	3	4	3
4	Mixture 1	Chitosan hydrochloride + sweet orange essential oil		300 + 400 mL/hL	7	3	5	3	3	4	3
5	Mixture 2	Chitosan hydrochloride + *Equisetum arvense*		300 + 400 mL/hL	7	3	5	3	3	4	3
6	Serenade Aso®	*Bacillus amyloliquefaciens* QST 713	Bayer Cropscience S.r.l., Milano, Italy	400 mL/hL	-	3	-	3	-	-	3
7	Kop-Twin[a]	Tribasic copper sulphate and copper hydroxide	Chimiberg - DIACHEM S.p.A., Caravaggio, Italy	350 g/hL	7	3	5	3	3	4	3

[a] Copper formulate was used as standard copper-based fungicide.

Orchard III, disease incidence and severity were recorded six times during 2019/20, five times in 2020/21, and four times in 2021/22.

For each treatment, disease incidence (DI) was visually assessed by calculating the percentage (%) of infected fruits within the four replicates. Each replicate consisted of four-six plants from which 50 fruits were assessed, for a total of 200 fruit per treatment. Disease severity (DS) was recorded as the percentage of symptomatic fruit area classified according to a six classes rating scale of damages on the fruit as follows: class 0 = no symptoms; class 1 = a few scattered lesions covering 1–5% of the fruit surface; class 2 = a few scattered lesions covering 6–10% of the fruit surface; class 3 = lesions covering 11–25% of the fruit surface; class 4 = extensive lesions covering 26–50% of the fruit surface; class 5 = severe lesions covering >50% of the fruit surface.

Moreover, the disease infection index (or McKinney's Index, DSI), which combines both incidence and severity of the disease, was expressed as a percentage according to the following equation:

$$\mathrm{DSI} = \frac{\sum (\mathrm{d} \times \mathrm{f})}{\mathrm{N} \times \mathrm{D}} \times 100 \tag{1}$$

where *d* is the category of disease class scored for citrus fruit; *f* is the disease frequency; *N* is the total number of examined fruit (healthy and symptomatic); and *D* is the highest class of disease intensity that occurred in the empirical scale (McKinney, 1923).

The efficacy of the alternative products on diseases symptoms reduction was calculated based on the equation proposed by Abbott (1925):

$$Efficacy = \frac{valC - valT}{valC} \times 100 \tag{2}$$

where *valC* and *valT* represent infection' values expressed as disease incidence (or disease severity) of the untreated control and of each treatment, respectively. The use of efficacy values allowed to compare each treatment with the untreated control and with standard copper fungicide included in the same experiment so to identify product equally or more active than copper.

Then, to determine if synergism was present in the effectiveness of mixtures, Limpel's formula (Richter, 1987) was applied as following:

$$Ee = X + Y - \frac{XY}{100} \tag{3}$$

where *Ee* is the expected effect from additive responses of two treatments, *X* and *Y* are the percentages of disease incidence or disease severity reduction relative to each alternative product used alone. Thus, if the combination of the two alternative products produces any value of symptoms reduction greater than *Ee*, then synergism exists.

2.6. Cost/benefit analysis of the alternative products

A balance sheet has been constructed with operational and actual accounting data, inferred using the "Cost Accounting System" approach (Sgroi et al., 2015; Lu et al., 2016; Berta et al., 2021). Since the objective of the research was to evaluate the effectiveness of the alternative plant protection products compared to the untreated control and Cu-based product, the economic balance only considered the operating costs, leaving out the fixed costs, which equally affect the alternative treatments' cost.

The analysis of the costs of the treatments and the benefits offered by alternative product was carried out assuming the following parameters obtained as average values from information provided by commercial operators active in the area: average production: 20,000 kg/ha; average production value: 0.35 Euro/kg; average spray volume: 2000 L/ha. For each alternative product, the following items were ascertained and calculated: cost of product (Euro/L); dose (mL of product/hL); applied dose (L of product/ha); cost of applied dose (cost of product × applied dose); cost of treatment (cost of applied dose × no. of applications each year). The following items were then calculated: damaged production (kg/ha); marketable production (kg/ha); economic value of the marketable production (Euro/ha); net economic value of the marketable production (Euro/ha) (i.e. economic value of the marketable production minus the cost of the treatment); net economic value of the marketable production compared with the untreated control (Euro/ha); increase (%) of net economic value of the marketable production versus the untreated control (net value increase vs untreated control). Three field trials were examined, corresponding to the second growing season for each orchard, and were characterized by different citrus accessions ('Femminello Siracusano 2 KR', 'Tarocco Tapi', 'Tarocco Scirè'), disease pressures (low, moderate, high), plant pathogens (*A. alternata* and *Colletotrichum* spp.) and number of treatments' applications (3, 3, 4).

2.7. Statistical analysis

Statistical analyses were performed using Minitab software (Minitab™, v. 20.1). The number of replicates used for statistical analysis was consistently organized using the uniform described methodology for each orchard by using four replicates for each treatment (each consisting of 200 fruits). All data were checked for normal distribution by Shapiro-Wilk normality test, considering a significance level (*p*-value) of 0.05. To evaluate data homogeneity, Levene's test at *p*-value<0.05 was employed. Not normalized data were transformed using arcsine ($\sin^{-1}$ square root *x*) prior to statistical analysis to make their distribution normal. To evaluate the effects of the factors "treatment", "citrus host" and their interaction on disease parameters, two-way Analysis of Variance (ANOVA) was performed at *p*-value<0.05. To assess the overall effects of the independent factor "treatment" on multiple dependent variables simultaneously considered ("disease incidence" and "disease severity"), Multivariate Analysis of Variance (MANOVA) was conducted on the whole dataset from the seven experimental trials. It was applied a significance level (*p*-value) of α = 0.05 to assess the statistical significance of the MANOVA results. To analyse the effect of treatments on single experiment, mean data for each parameter (disease incidence, disease severity and disease severity index) were subjected to one-way ANOVA. Differences among treatments were analysed by Fisher's least significant differences (LSD) post hoc test. A *p*-value of <0.05 was considered statistically significant.

3. Results

Four alternative products, alone and in mixture, for a total of six treatments, were evaluated under field conditions in three orchards located in different agricultural areas in south-eastern Sicily (Agnone, Mineo and Pedagaggi), on different citrus species and accessions (lemon 'Femminello Siracusano 2 KR', oranges 'Tarocco Tapi' and 'Tarocco Scirè') and in different growing seasons to control ABS (in Orchard I) and anthracnose (in Orchards II and III) on fruit caused by *Alternaria* and *Colletotrichum* species, respectively. The products' efficacy was compared with untreated control and copper reference treatments. Differences in disease incidence in untreated control, assessed in each orchard and year at the last monitoring time, coinciding with harvest time, led to identify three different scenarios of disease pressure: low, intermediate and high. In Orchard I (lemon 'Femminello Siracusano 2 KR'), ABS infections caused intermediate (32% in 2020) and low (11.7% in 2021) levels of disease incidence. Anthracnose symptoms on 'Tarocco' blood oranges caused by *Colletotrichum* spp. induced low (7.5% in 2020) and intermediate (34.5% in 2021) levels of disease incidence in Orchard II (orange 'Tarocco Tapi'), and high (45%, 69.3% and 40%, during the three growing seasons) incidence levels in Orchard III (orange 'Tarocco Sciré') (Supplementary Fig. S2).

Two-way ANOVA confirmed these results, and revealed that "citrus host" comprehensively exerted a significant influence on disease parameters (DI, $F_{2,184} = 27.68$, *p*-value<0.001, DS, $F_{2,184} = 26.21$, *p*-value<0.001, Supplementary Table S2). Similarly, "treatments" significantly impacted on disease incidence and disease severity (DI, $F_{7,184} = 8.83$, *p*-value<0.001, DS, $F_{2,184} = 9.04$, *p*-value<0.001, Supplementary Table S2), while the interaction between "treatment" and "citrus host" did not exhibit statistical significance (*p*-value>0.05).

Multivariate Analysis of Variance (MANOVA), conducted on the entire dataset, evidenced a significant influence of the independent factor "treatment" on the dependent variables "disease incidence" and "disease severity" simultaneously (Pillai's V = 0.23, $F_{7,200} = 3.75$, *p*-value<0.001, $\eta p^2 = 0.12$, Supplementary Fig. S3). Subsequent analysis for each dependent variable indicated that each applied treatment significantly influenced both diseases incidence and severity, being statistically different from untreated control values (Supplementary Fig. S3).

The results were further explored for each field experiment carried out in the Orchards I, II and III.

3.1. Evaluation of Alternaria brown spot infection on fruit in Orchard I

Brown spot symptoms on fruit were widely detected in lemon 'Femminello Siracusano 2 KR' in orchard I during the two growing seasons (Supplementary Fig. S4), even though disease incidence progressed differently over time in the assessed years, reasonably due to different climatic conditions.

During 2020, in Orchard I (lemon 'Femminello Siracusano 2 KR') spring temperatures below 17 °C and abundant rainfall in April–May (Fig. 1A) induced extended periods of leaf wetness, that were also maintained due to rainfall that occurred in July, together with optimum temperature for the infection (25 °C).

The described weather conditions were favourable for the onset of ABS infections on immature fruits during late summer. Then, the rainfalls that occurred from September through November 2020 were favourable for ABS development on ripening fruit too.

During the first growing season, first symptoms of ABS appeared on lemon fruit late in July (concurrent to the first treatment), and the disease incidence was very low (<2%) (data not shown). In the following assessments, an increase in the ABS incidence was observed, reaching the 32% in untreated control during the last survey (November 10, 2020) before harvest (Fig. 2A).

At the last evaluation time, all treatments reduced the incidence and severity of ABS on fruits with significant differences among products (Table 2). Specifically, lemon fruits treated with chitosan hydrochloride, *E. arvense*, sweet orange essential oil, Mixture 1 (chitosan hydrochloride + sweet orange essential oil) and Mixture 2 (chitosan hydrochloride + *E. arvense*) reduced both disease incidence (56.3, 68.8, 65.6, 78.1 and 81.3, according to Abbott's formula), and disease severity index (52.4, 73.7, 65.5, 77 and 78.6%) when compared to untreated control, while copper only reached a reduction of 43.8% of disease incidence and 32.8% of McKinney's index. Noteworthy, Mixture 1 and Mixture 2 showed to be the most effective treatments, with significantly better results than those obtained by copper-standard fungicide, whereas the remaining treatments showed comparable results to copper. Moreover, copper-treatment didn't show a significant reduction of disease severity when compared to untreated control.

During the second year (2021), the unfavourable climatic conditions for disease development, represented by prolonged arid periods and high temperatures in summer, led to a low incidence of ABS during the whole season. However, the heavy rainfalls that occurred from September to November, with highest values intercepted in October, were more conducive for the late onset of disease. In fact, in the last assessment (Fig. 2B), an increase in the disease incidence on fruit was observed, up to 11.7% in untreated control during the last survey (November 5, 2021), while disease incidence's values were lower in the treated fruits, ranging between 0.8 and 5.4% (Table 2). Overall, all alternative treatments significantly reduced disease incidence and severity if compared with untreated control (Table 2). When compared to copper-fungicide, sweet orange essential oil and Mixture 1 achieved a significantly better disease control, whereas the remaining treatments showed a comparable efficacy than those obtained by copper treatments. Additionally, the combination of the products in Mixture 1 (chitosan hydrochloride + sweet orange essential oil) evidenced a synergistic activity, since the observed effect is greater than the expected effect (Ee), according to Limpel's formula (Table 2).

3.2. Evaluation of preharvest anthracnose infection on fruit in Orchard II

In Orchard II (orange 'Tarocco Tapi'), during the first year (2020), the abundant rainfalls in March, July and November, associated with an increase in temperature, represented favourable conditions for the onset of the anthracnose infection on citrus fruits (Fig. 1B). Nevertheless, the low disease pressure at the end of the growing season (up to 7.5 % on November 10, 2020) could reasonably be due to the citrus pruning carried out in February, that reduced the inoculum load in the plants and so reduced the impact of anthracnose symptoms.

During the second year (2021), rainfalls in spring and summer periods, coincident with an increase in temperature, and abundant rainfalls in October represented favourable climatic conditions for disease development on fruits (Supplementary Fig. S5) that led to higher incidence of anthracnose on fruit (34.5%) on 18 November, before harvesting time.

In 2020 trial, the first symptoms of anthracnose appeared on sweet orange fruit 'Tarocco Tapi' on late July, with a very low (<1%) disease incidence (data not shown). In the following assessments, a moderate increase of disease incidence values was recorded (Fig. 2C), reaching the highest value on untreated control before harvesting time, on November 10, 2020 (DI 7.5%).

Even though disease incidence's values were low in all treated fruits at the last monitoring time, ranging between 1.3 and 4.8%, statistical differences among treatments were recorded (Table 3). Specifically, if compared to untreated control, disease incidence was significantly reduced by *E. arvense*, sweet orange essential oil, Mixture 1 (chitosan hydrochloride + sweet orange essential oil) and Mixture 2 (chitosan hydrochloride + *E. arvense*) (82.6, 62.6, 69.3, and 73.3% respectively), whereas in fruit treated with chitosan hydrochloride no significant differences were recorded. Moreover, all treatments significantly reduced disease severity as well as disease severity index. Treatments reduced disease severity index by 44.4, 83.3, 61.1, 66.6 and 72.2%, respectively for chitosan hydrochloride, *E. arvense*, sweet orange essential oil, Mixture 1, Mixture 2. When compared to copper-standard fungicide, that reached 62.6% of DI reduction and 66.7% of DSI reduction, significant better results in disease incidence reduction were achieved by *E. arvense*, whereas all the remaining alternative products showed a comparable control efficacy of both incidence and severity.

In the second year (2021), first symptoms of preharvest anthracnose on fruit appeared in late August, with values below 2% (data not shown). In the following disease assessments (Fig. 2D), a significant increase in the DI levels of anthracnose on untreated fruit was recorded, with the highest values during the last survey on 18 November (DI up to 34.5%), while the DI among the alternative treatments ranged from 12.2 to 29.5% (Table 3). Chitosan hydrochloride, sweet orange essential oil, Mixture 1 (chitosan hydrochloride + sweet orange essential oil), Mixture 2 (chitosan hydrochloride + *E. arvense*) and *B. amyloliquefaciens* QST 713 significantly reduced DI compared with untreated control by 64.7, 46.4, 58.9, 39.6, and 34.3%, respectively (Table 3), as well as disease severity index (70, 60.7, 66.1, 45.3 and 42.3% of reduction, respectively). On the opposite, in fruit treated with *E. arvense* and copper, DI, DS and DSI were not significantly different from untreated control. Thus, all treatments, except for *E. arvense*, were more effective than copper.

3.3. Evaluation of preharvest anthracnose infection on fruit in Orchard III

In Orchard III (orange 'Tarocco Scirè'), over the three years of trials,

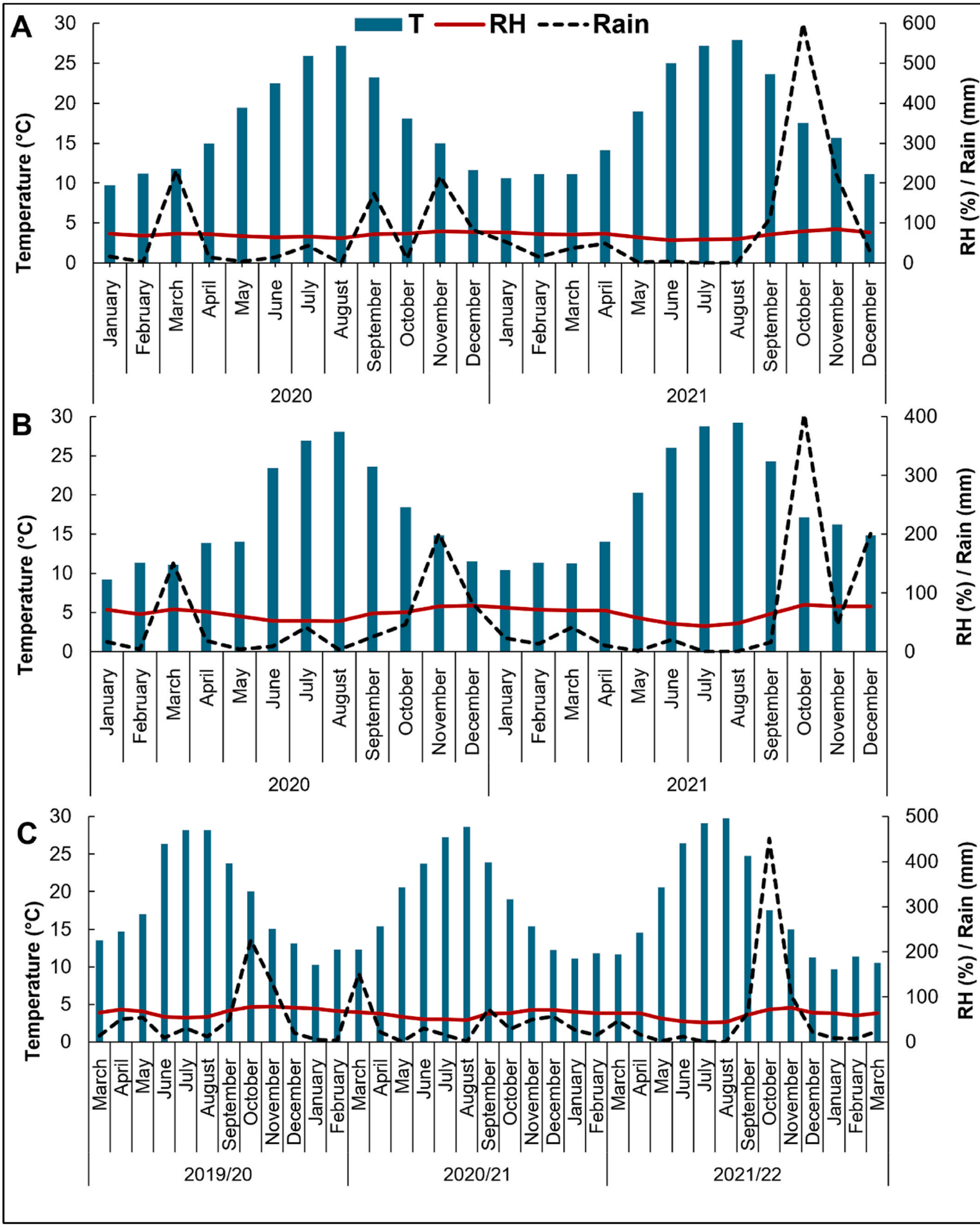

Fig. 1. Rain (mm), temperature (°C) and relative humidity (%) recorded by the weather station of (A) Augusta (Syracuse), (B) Mineo (Catania) and (C) Francofonte (Syracuse) during the growing seasons. T = Temperature; RH= Relative humidity.

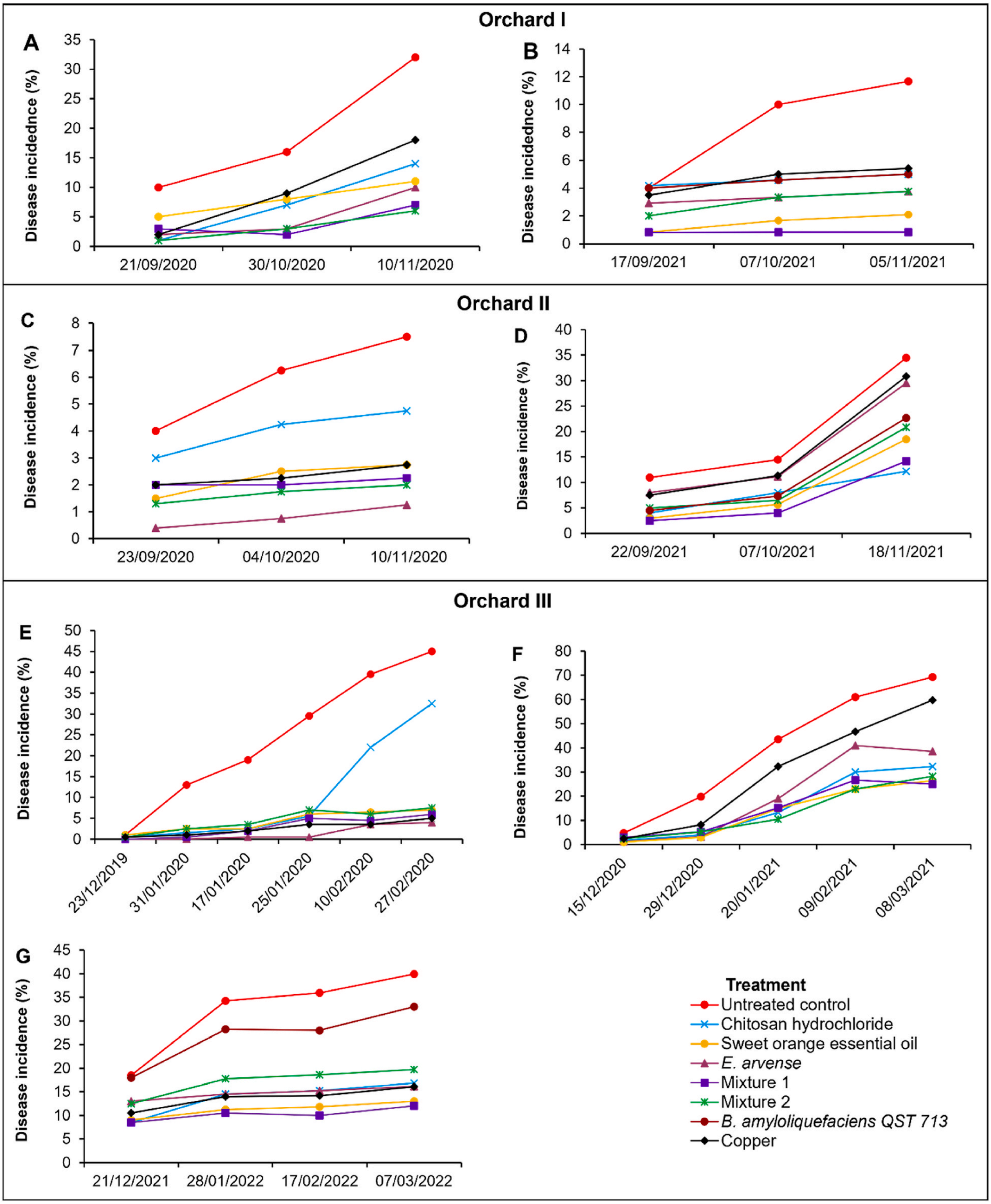

Fig. 2. Time progression of Alternaria brown spot (ABS) symptoms on lemon fruit 'Femminello Siracusano 2 KR' (Orchard I) during first (A) and the second (B) growing season; of anthracnose on orange fruit 'Tarocco Tapi' (Orchard II) during the first (C) and the second (D) growing season; of anthracnose on orange fruit 'Tarocco Scirè (Orchard III) during the first (E), the second (F) and the third (G) growing season. Field treatments consisted in application of alternative products and copper. Disease incidence was expressed as percentage of symptomatic fruits/treatment.

symptoms of anthracnose consisting in dry and rounded depressed lesions were observed (Supplementary Fig. S6) and severe infections occurred with cool, moist winters and dry summers, so then high disease incidence values were recorded. Specifically, during the first trial (2019/20), abundant rainfalls during the autumn followed by a cool and moist winter (Fig. 1C) were favourable to the onset and spread of the disease, leading to high DI on untreated control (45%) at the end of February 2020, before harvesting time.

During the second trial (2020/21), climatic conditions characterized by moist autumn and cool winter, that coupled with hailstorm damages

Table 2
Disease incidence (DI), severity (DS) and McKinney's index (MI) of Alternaria brown spot (ABS) infections recorded on lemon fruit 'Femminello Siracusano 2 KR' in Orchard I on November 10, 2020 (first trial) and on November 5, 2021 (second trial), corresponding to the last monitoring time, following the treatments with different products during the two seasons*.

Treatment	Orchard I – 'Femminello Siracusano 2 KR'					
	First Trial – 2020			Second Trial – 2021		
	DI (%)	DS (0–5)	DSI (%)	DI (%)	DS (0–5)	DSI (%)
Untreated control	32.0 ± 5.6 a	0.61 ± 0.13 a	12.2 ± 2.6 a	11.7 ± 0.6 a	0.29 ± 0.12 a	5.7 ± 0.8 a
Chitosan hydrochloride	14.0 ± 2.0 b	0.29 ± 0.03 bc	5.8 ± 0.7 bc	5.0 ± 0.9 bc	0.10 ± 0.10 bc	1.9 ± 0.7 bc
E. arvense	10.0 ± 2.0 bc	0.16 ± 0.03 c	3.2 ± 0.6 c	3.7 ± 0.6 bc	0.06 ± 0.06 bcd	1.3 ± 0.4 bcd
Sweet orange essential oil	11.0 ± 1.0 bc	0.21 ± 0.04 bc	4.2 ± 0.9 bc	2.1 ± 0.5 cd	0.03 ± 0.04 cd	0.6 ± 0.3 cd
Mixture 1	7.0 ± 3.4 c	0.14 ± 0.07 c	2.8 ± 1.4 c	0.8 ± 0.5 d**	0.01 ± 0.04 d	0.3 ± 0.3 d
Mixture 2	6.0 ± 2.6 c	0.13 ± 0.05 c	2.6 ± 1.0 c	3.7 ± 0.6 bc	0.06 ± 0.04 bcd	1.2 ± 0.3 bcd
B. amyloliquefaciens QST 713	-	-	-	5.0 ± 0.4 b	0.09 ± 0.03 bc	1.8 ± 0.2 bc
Copper	18.0 ± 2.6 b	0.41 ± 0.1 ab	8.2 ± 2.1 ab	5.4 ± 0.6 b	0.10 ± 0.03 b	2.0 ± 0.4 b

*Data presented as means (±SEM) of 4 replicates (each consisting of 50 fruits) followed by the same letter indicate no difference among the tested products according to Fisher's least significance difference test (LSD) at *p*-value< 0.05.
**indicates a synergistic activity according to Limpel's formula.

Table 3
Disease incidence (DI), severity (DS) and disease severity index (DSI) of citrus anthracnose infection recorded on orange fruit 'Tarocco Tapi' in Orchard II on November 10, 2020 (first trial) and November 18, 2021 (second trial), corresponding to the last monitoring time, following the treatments with the treatments with the different products during the two seasons*.

Treatment	Orchard II - 'Tarocco Tapi'					
	First Trial - 2020			Second Trial – 2021		
	DI (%)	DS (0–5)	DSI (%)	DI (%)	DS (0–5)	DSI (%)
Untreated control	7.5 ± 1.6 a	0.09 ± 0.02 a	1.8 ± 0.4 a	34.5 ± 3.6 a	0.7 ± 0.08 a	13.0 ± 1.6 a
Chitosan hydrochloride	4.8 ± 0.5 ab	0.05 ± 0.01 b	1.0 ± 0.1 b	12.2 ± 1.4 c	0.2 ± 0.03 d	3.9 ± 0.6 d
E. arvense	1.3 ± 0.8 d	0.02 ± 0.01 c	0.3 ± 0.2 c	29.5 ± 2.1 a	0.5 ± 0.06 a	10.7 ± 1.1 a
Sweet orange essential oil	2.8 ± 0.3 bc	0.03 ± 0.0 bc	0.7 ± 0.1 bc	18.5 ± 1.4 b	0.3 ± 0.03 bcd	5.1 ± 0.6 bcd
Mixture 1	2.3 ± 0.3 c	0.03 ± 0.0 bc	0.6 ± 0.1 bc	14.2 ± 2.1 c	0.2 ± 0.04 cd	4.4 ± 0.8 cd
Mixture 2	2.0 ± 0.4 cd	0.02 ± 0.0 bc	0.5 ± 0.1 bc	20.8 ± 2.1 b	0.4 ± 0.05 bc	7.1 ± 1.0 bc
B. amyloliquefaciens QST 713	-	-	-	22.7 ± 2.4 b	0.4 ± 0.05 b	7.5 ± 1.1 b
Copper	2.8 ± 0.3 bc	0.03 ± 0.0 bc	0.6 ± 0.1 bc	30.8 ± 2.3 a	0.6 ± 0.06 a	11.9 ± 1.3 a

*Data presented as means (±SEM) of 4 replicates (each consisting of 50 fruits) followed by the same letter indicate no difference among the tested products according to Fisher's least significance difference test (LSD) at *p*-value< 0.05.

and rainfalls in December–January, prolonged the fruits wetness, and with high humid conditions, were favourable to a severe anthracnose symptoms development on fruits and a more severe incidence (DI 69.3%). While, during the third trial (2021/22), hot-dry summer and heavy rainfall in autumn and winter were favourable to the onset and spread of the disease, which increased up to 40% (DI) at the end of February, before harvest.

In the first trial (2019/20), in the assessments carried out from 23 December to 27 February (Fig. 2E), an increase in the disease incidence level of anthracnose symptoms on untreated fruit was recorded, with the highest values (DI 45%) at the last monitoring time, before harvest.

In treated fruits, DI ranged between 4 and 32.5%, and statistical differences among treatments were observed (Table 4). Compared to the untreated control, DI was significantly reduced by all treatments (chitosan hydrochloride by 27.8%, *E. arvense* by 91%, sweet orange essential oil by 84.4%, Mixture 1 by 86.7 and Mixture 2 by 83.3%), as well as DS values (Table 4). Similarly, the DSI values were significantly reduced by the aforementioned treatments by 35.7, 94.9, 89.5, 90.6, 88.4 and 93.5%, respectively, if compared to untreated control. When compared to copper-standard fungicides, all treatments showed a comparable effectiveness, with the only exception of chitosan hydrochloride.

In the second trial (2020/21), the first symptoms of anthracnose appeared on citrus fruit on late November, and low levels of disease incidence (below 1%) were recorded (data not shown). In the following assessments (Fig. 2F), an increase of disease incidence levels was recorded on untreated fruits, from 20% during the second survey of 29 December, up to 69% at the last survey (8 March), with a percentage increase of +245%. Although at high disease pressure in untreated fruits at the last monitoring time, disease incidence of treated fruits ranged between 25 and 38.5 % (Table 4). Citrus treated with chitosan hydrochloride, *E. arvense*, sweet orange essential oil, Mixture 1 (chitosan hydrochloride + sweet orange essential oil) and Mixture 2 (chitosan hydrochloride + *E. arvense*) showed a significant reduction in anthracnose symptoms by 53.4, 44.4, 61.7, 63.9, and 59.2%, respectively, as well as disease severity was significantly reduced by all treatments (Table 4). Disease severity index showed similar trends and a reduction of 61.9, 52.8, 64.2, 66.4 and 64.2%, respectively to the previously mentioned treatments. During this trial, copper showed to be ineffective in reducing preharvest anthracnose of citrus fruit, recording DI, DS and DSI values which did not differ significantly from untreated control.

As well as the previous growing seasons, in the third year (2021/22), first anthracnose symptoms appeared on fruit in late November, with disease incidence values of approximately 2% (data not shown), and an increase on disease incidence values was recorded in the following assessments (Fig. 2G). Even under a more moderate disease pressure up to 40% at the last survey on untreated control, all the products reduced the disease incidence in the range of 12 and 33% (Table 4). Specifically, disease incidence was significantly reduced by chitosan hydrochloride and *E. arvense* by 57.7%, sweet orange essential oil by 67.2%, Mixture 1 by 69.3% and Mixture 2 by 48.2% (Table 4). Disease severity index was reduced as well, when compared to untreated control by 71.6, 75.3, 72.3, 79.8, 57.4 and 67.5%, respectively. On the opposite, *B. amyloliquefaciens* QST 713 was not effective on reducing natural infection caused by *Colletotrichum* spp. on oranges 'Tarocco Scirè'. Moreover, all alternative treatments showed to be as effective as copper treatments (Table 4).

Table 4
Disease incidence (DI), severity (DS), and disease severity index (DSI) of citrus anthracnose infections recorded on orange fruit 'Tarocco Sciré' in Orchard III on February 27, 2020 (first trial), March 8, 2021 (second trial) and March 7, 2022 (third trial), corresponding to the last monitoring time, following the treatments with the different products during the three seasons*.

Treatment	Orchard III – 'Tarocco Scirè'								
	First Trial - 2019/20			Second Trial - 2020/21			Third Trial - 2021/22		
	DI (%)	DS (0–5)	DSI (%)	DI (%)	DS (0–5)	DSI (%)	DI (%)	DS (0–5)	DSI (%)
Untreated control	45.0 ± 4.3 a	1.4 ± 0.19 a	27.7 ± 3.9 a	69.3 ± 6.9 a	1.8 ± 0.2 a	35.2 ± 4.8 a	39.9 ± 5.9 a	1.3 ± 0.2 a	26.8 ± 3.8 a
Chitosan hydrochloride	32.5 ± 6.9 b	0.9 ± 0.24 b	17.8 ± 4.7 b	32.3 ± 8.7 c	0.7 ± 0.3 c	13.4 ± 5.3 c	16.9 ± 2.7 bc	0.4 ± 0.0 c	7.6 ± 0.8 c
E. arvense	4.0 ± 1.4 c	0.1 ± 0.03 c	1.4 ± 0.6 c	38.5 ± 9.5 bc	0.8 ± 0.2 c	16.6 ± 4.1 bc	16.1 ± 1.6 bc	0.6 ± 0.0 bc	6.6 ± 1 c
Sweet orange essential oil	7.0 ± 1.9 c	0.1 ± 0.05 c	2.9 ± 1.1 c	26.5 ± 7.3 c	0.6 ± 0.2 bc	12.6 ± 4.5 c	13 ± 2.5 c	0.3 ± 0.0 c	7.4 ± 0.4 c
Mixture 1	6.0 ± 1.2 c	0.1 ± 0.03 c	2.6 ± 0.6 c	25.0 ± 6.0 c	0.6 ± 0.2 c	11.8 ± 3.4 c	12.0 ± 3 c	0.3 ± 0.0 c	5.4 ± 0.9 c
Mixture 2	7.5 ± 0.9 c	0.2 ± 0.04 c	3.2 ± 0.9 c	28.3 ± 6.7 c	0.6 ± 0.2 c	12.6 ± 3.9 c	19.8 ± 3.3 bc	0.6 ± 0.1 bc	11.4 ± 1.2 bc
B. amyloliquefaciens QST 713	-		-	-	-	-	33.0 ± 9.4 ab	1.0 ± 0.2 ab	20.4 ± 4.2 ab
Copper	5.0 ± 1.3 c	0.1 ± 0.03 c	1.8 ± 0.5c	59.8 ± 10.1 ab	1.4 ± 0.2 ab	28.1 ± 4.9 ab	16.1 ± 4.8 bc	0.4 ± 0.1 bc	8.7 ± 2.3 bc

*Data presented as means (±SEM) of 4 replicates (each consisting of 50 fruits) followed by the same letter indicate no difference among the tested products according to Fisher's least significance difference test (LSD) at *p*-value< 0.05.

3.4. Cost/benefit analysis of the alternative products

Three of the seven field trials, chosen for different variables (disease pressure, growing season, accession, plant pathogens and number of treatments' applications), were subjected to a cost/benefit analysis of the alternative products' application.

In the three examined trials (Table 5), the efficacy of the treatments in reducing anthracnose and ABS influenced the total marketable production of lemon 'Femminello Siracusano 2 KR' (Orchard I) and oranges 'Tarocco Tapi' (Orchard II) and 'Tarocco Scirè' (Orchard III). Specifically, under conditions of high disease pressure (DI 69.3% in Orchard III during the second growing season), on high susceptible orange 'Tarocco Scirè', the total marketable production obtained treating with alternative products ranged between 12,300 and 15,000 kg/ha, almost double than in untreated control (6150 kg/ha) and in copper treatment (8050 kg/ha). Moreover, all the treatments obtained an increase of net economic values if compared to the untreated control, variable from 21.6 (Mixture 2) to 100.4% (sweet orange essential oil-based Prev-Am Plus), largely higher than that obtained with copper (14.3%). Their effectiveness and convenience were thus demonstrated: the high number of applications (4) and the relative high cost of the treatments were somewhat offset by the increased production and by the increases of net economic values of the production when compared to both untreated control and copper. Even under an intermediate (DI 34.5% in Orchard II during the second growing season) and a low pressure condition (DI 11.67% in Orchard I during the second growing season), the total marketable productions in treated plots were higher than untreated control and copper, and then, their economic values were similar or higher than those of copper treated plots (Table 5). Under an intermediate disease pressure on moderately susceptible orange 'Tarocco Tapi' (Orchard II), Chitosano Biorend, Prev-Am Plus and *B. amyloliquefaciens* QST 713 showed a good level of protection, a good net economic value of the marketable production and a higher increase of net economic values (21.2, 10.8 and 9.1%, respectively) compared to copper (0.1%). Finally, in low pressure conditions of the disease caused by *A. alternata* (Orchard I) on lemon 'Femminello Siracusano 2 KR', the significant reduction of DI was not correlated with an increase of net economic values of the production, and the cost of the treatments was equal to or greater than the recorded benefits.

4. Discussion

To our knowledge, this study provides for the first time a field validation in pluriannual trials of the efficacy of four commercially available alternative products, alone and in mixture, against anthracnose caused by *Colletotrichum* spp. and ABS caused by *A. alternata* on citrus fruits. The primary objective was to identify viable alternatives to traditional copper fungicides, with chief aim of safeguarding the quality of organic citrus production in Sicily. The efficacy of alternative products was examined on different citrus species (lemon and oranges) and clones ('Femminello Siracusano 2 KR', 'Tarocco Tapi' and 'Tarocco Scirè') in three commercial citrus orchards located in south-east Sicily (Italy). Overall, all the alternative products showed to reduce disease values in different scenarios, often showing similar efficacy to Cu-based compound.

Specifically, our results proved for the first time the field efficacy of chitosan hydrochloride, a natural biodegradable polymer obtained from chitin, alone and in mixture, towards *A. alternata* on lemon fruits, widening the limited available information on its control effects in situ (open field). Our previous results were also confirmed on blood orange 'Tarocco' against *Colletotrichum* spp. Formerly, the direct fungicidal activity of chitosan against the causal agents of anthracnose and ABS has been mostly documented on laboratory screening (Deng et al., 2015a, 2015b; Garganese et al., 2019; Zhao et al., 2018; Zhou et al., 2016; Zivkovic et al., 2018). Only occasionally its efficacy has been reported in open field conditions against ABS and anthracnose, considering other plant hosts (Feliziani et al., 2015; Lutz et al., 2022; Toffolatti et al., 2023; However, Garganese et al., 2019 was the sole documentation in literature for citrus crop, applied in mandarin. The great potential of chitosan as an antifungal preservative is attributable not only to the direct fungicidal activity, but to an indirect activity too, acting as elicitor of response mechanisms in plants, and film-forming properties (Ali et al., 2010, 2013; Romanazzi et al., 2018), and its mechanisms on orange navel has been elucidated (Deng et al., 2015a, 2015b; Zhao et al., 2018).

Similarly, results revealed that sweet orange essential oil, a complex mixture of volatile and non-volatile compounds, significantly reduced disease parameters, often resulting in a better disease control compared to copper-standard fungicide, thus confirming our preliminary results (Lombardo et al., 2023). The antifungal properties of sweet orange essential oil have been previously reported in agar plates against major and minor citrus pathogens (Regnier et al., 2014; Tao et al., 2013), including *C. gloeosporioides* and *A. alternata* (Almada-Ruiz et al., 2003; Bosquez-Molina et al., 2010; Chutia et al., 2009; Phillips et al., 2012; Singh et al., 2010), but no attempts have been made on citrus fruits or in semi-commercial conditions so far. Importantly, the antifungal activity of sweet orange essential oil may be primarily attributed to their main components as well as to the interaction effect of major and minor components, resulting in low possibility for fungal pathogens to develop resistance (Jing et al., 2014; Bakkali et al., 2008, Da Costa Gonçalves et al., 2021), avoiding critical side effects typically associated with the use of chemical compounds (Gama et al., 2020; Dewdney, 2023; Chitolina et al., 2021). Taken together, these findings suggested that sweet

Table 5
Cost of treatments, marketable productions, and increases of economic values compared to untreated control.

Citrus orchard*	Treatment**	DI (%)	Cost of product (Euro/L)	Dose (mL/hL)	[a]Applied dose (L/ha)	[b]Cost of applied dose (Euro/ha)	[c]Cost of treatment in each year (Euro/ha)	[d]Damaged production (kg/ha)	[e]Marketable production (kg/ha)	[f]Economic value of marketable production (Euro/ha)	[g]Net economic value of the marketable production (Euro/ha)	[h]Net economic value vs control (Euro/ha)	[i]Increase of net economic value vs control (%)
Orchard I	Control	11.7						2333	18	6,2			
	Chitosano Biorend	5	32.9	300	6	197.4	591	1	19	6,7	6059	−124	−2
	Prev-Am Plus	2.1	26	400	8	208	624	417	20	6,9	6,23	47	0.8
	Naturdai Equibasic	3.8	22	400	8	176	528	750	19	6,7	6,21	26	0.4
	Mixture 1	0.8	59	700	14	405	1215	167	20	6,9	5727	−457	−7.4
	Mixture 2	3.8	55	700	14	373	1119	750	19	6,7	5619	−565	−9.1
	Serenade	5	17	400	8	136	408	1	19	6,7	6242	59	0.9
	Kop-Twin	5.4	12	350	7	89.4	268	1083	19	6,6	6353	169	2.7
Orchard II	Control	34.5						6900	13	4,6			
	Chitosano Biorend	12.2	32.9	300	6	197.4	591	2433	18	6,1	5557	972	21.2
	Prev-Am Plus	18.5	26	400	8	208	624	3700	16	5,7	5081	496	10.8
	Naturdai Equibasic	29.5	22	400	8	176	528	5900	14	4,9	4407	−178	−3.9
	Mixture 1	14.2	59	700	14	405	1215	2833	17	6	4793	208	4.5
	Mixture 2	20.8	55	700	14	373	1119	4167	16	5,5	4423	−162	−3.5
	Serenade	22.7	17	400	8	136	408	4540	15	5,4	5003	418	9.1
	Kop-Twin	30.8	12	350	7	89.4	268	6167	14	4,8	4573	−12	0.3
Orchard III	Control	69.3						13,85	6	2,2			
	Chitosano Biorend	32.3	32.9	300	6	197.4	788	6,45	14	4,7	3955	1802	83.7
	Prev-Am Plus	26.5	26	400	8	208	832	5,3	15	5,1	4313	2161	100.4
	Naturdai Equibasic	38.5	22	400	8	176	704	7,7	12	4,3	3601	1449	67.3
	Mixture 1	25	59	700	14	405	1620	5	15	5,3	3,63	1478	68.6
	Mixture 2	28.3	55	700	14	373	1492	5.65	14	5	3531	1378	21.6
	Kop-Twin	59.8	12	350	7	89.4	357	11,95	8	2,8	2,46	307	14.3

*Benefit/cost analysis is showed for the second growing season for each orchard: in Orchard I and II during 2021 fruits has been treated three times, in Orchard III during 2020/2021 fruits has been treated four times.
**Commercial products are listed as trade name.
[a] Applied dose = dose × 2000 L.
[b] Cost of applied dose = cost of product × applied dose; values of mixtures are the sum of the cost of the single treatment.
[c] Cost of treatment = cost of applied dose × no. of applications each year.
[d] Damaged production = mean production (20,000 kg/ha) × DI (%)/100.
[e] Marketable production = mean production - damaged production.
[f] Economic value of marketable production = marketable production × 0.35 (mean value of 1 kg of citrus).
[g] Net economic value of marketable production = Economic value of the marketable production - cost of treatment.
[h] Net economic value vs control = Net economic value of the marketable production - Economic value of the marketable production of the control.
[i] Increase of net economic value vs control = Net economic value vs control/Economic value of the marketable production of the control × 100.

orange essential oil have the potential to be an efficient alternative to replace standard fungicides in the management of preharvest anthracnose or ABS of citrus fruit.

In our previous work, the direct antifungal efficacy of *E. arvenses* was proved against *C. gloeosporioides* in both *in vitro* and *in vivo* trials, and this study represent the first open-field report of *E. arvense* against citrus fungal pathogens, and more specifically against Colletotrichum and Alternaria diseases. *E. arvense* has been recently studied for its preventive effect on fungal pathogens attributable to the high percentage of silica (Marchand, 2016; García-Gaytán et al., 2019), commonly known to strengthen plant cell tissues as so acting as a physical barrier that prevents the penetration of the fungal appressorium into the plant (Fauteux et al., 2005; Marchand, 2016). In addition, the direct antifungal efficacy of *E. arvense* may be attributed to its high concentration of flavonoids and phenols (Pallag et al., 2016). Its antifungal activity has been demonstrated on a wide number of fungi of phytopathological interest (Dagostin et al., 2011; Marchand, 2016; Llamazares De Miguel et al., 2022; Trebbi et al., 2021). *E. arvenses* was also recently confirmed as a suitable Cu-alternative to manage tomato late blight, confirming its efficacy in the field over a three-year period experiment (Trebbi et al., 2021). Several commercial products containing field horsetail are already commercially available and registered for the control of plant pathogens, in accordance with SANCO/12386/2013 in Appendix II, thereby positioning *E. arvenses* a potentially suitable alternative. Moreover, its cost-effectiveness, compared to the other alternatives, along with the current registration on citrus crop as active substance, strengthens *E. arvense* as a potential copper substitute.

In addition, data indicated that the combination of chitosan with sweet orange essential oil (Mixture 1) and with *E. arvense* (Mixture 2) as commercial formulations could potentially provide a better effective pathogens' control compared with products applied alone. In fact, in some trials, products in mixture achieved the best disease reduction values, comprehensively due to higher rate of application than the product applied alone and then the total effect could be simply additive. In this regard, to claim that the mixtures exhibit synergism, further research are needed also to elucidate the factor of the possible interaction of substances in mixture. In literature, the synergistic effect of chitosan employed in combination with essential oil, instead of the stand-alone basic substance, in reducing the anthracnose of fruit has been evidenced (Grande-Tovar et al., 2018; Lima Oliveira et al., 2018; Peralta-Ruiz et al., 2020). It has been suggested that chitosan and essential oils can synergistically act to enhance the antifungal properties of each other; probably, chitosan affects the permeability of fungal membranes and reduces the synthesis of cell wall components, causing decreased ability of the target fungi to tolerate the disturbing effects of the essential oil's constituents on surface characteristics and fungal cell structure (Elsabee and Abdou, 2013). Still, the disturbing effects of chitosan on fungal cell structure may increase the partition of the essential oils' constituents into fungal cells, where they can act on target structures (Athayde et al., 2016; Santos et al., 2012). Moreover, the enhanced effect of chitosan-*E. arvense* mixtures was evidenced by Langa-Lomba et al. (2021), which reported a synergistic effect of the two substances in combination expressed through a significant reduction of vascular necrosis in grapevine plants caused by artificial inoculations of *Botryosphaeriaceae* spp., and some possible modes of action have been hypothesized.

In the present investigation, we also reported a first attempt to test the field efficacy of a bioformulate containing *B. amyloliquefaciens* QST 713 towards anthracnose on orange fruit 'Tarocco' and ABS on lemon fruit, and its control activity was demonstrated under low and intermediate disease pressure, whereas was ineffective under high disease pressure. These results further highlighted the importance of including multiple years and hosts when testing the efficacy of Cu alternatives under field conditions (He et al., 2021; Scortichini, 2022). Its worldwide utilization, due to the broad-spectrum, covers all kinds of fungal diseases in diverse crops (Luo et al., 2022; Fischer et al., 2013), acting by producing secondary metabolites that disrupt germ tube formation of pathogens, creating an inhibition zone, competing for space and nutrients and thus preventing pathogens spores from germinating. An extensive literature reported the biocontrol activities of *B. amyloliquefaciens* strains against diverse citrus diseases (Huang et al., 2012; Arrebola et al., 2010a; Aiello et al., 2022; Leonardi et al., 2023), including field experiments of commercial formulations on diverse symptoms caused by *Colletotrichum* spp. or *A. alternata* (Agostini et al., 2003). In fact, *Bacillus*-based biocontrol products is a promising area of study and, in the view of further surveys, it is worth to mention that the possibility of a combined strategy with chitosan, essential oil or other BCAs could improve the performance of *Bacillus* species (Ahmed et al., 2003; Arrebola et al., 2010b), and that some *Bacillus*-based formulations are compatible as a tank mix with many citrus fungicides, including copper.

As already discussed, the activity of alternative products was often similar or better than those obtained by copper applications. However, since copper-compounds' treatments are not free from side effect, an integrated pest management system with alternative formulations as the ones tested in our study could maintain a similar level of control without side effects, thus balancing the higher cost of alternative treatments' application (Scortichini, 2022; Toffolatti et al., 2023). Thus, the good results achieved by the tested alternative products against anthracnose and ABS of citrus fruit, demonstrated in different scenarios, strongly encourage research efforts in this direction. While waiting for further field trials to be conducted, it is necessary to recall other useful elements to positively reinforce the economic assessment: the average production, the average production value and the price system adopted (referred to the market for the supply of alternative products and the market for the placement of citrus production) has been considered homogeneous in the surveyed areas, and did not take into account possible variations on the local scale; the assessment can be extended to include social aspects (direct and indirect activities) and food security; a cost's reduction in the market of alternative products can be expected, as their use will increase in the next future and the possibility of collective purchase through the defence consortia active at territorial level (Singh et al., 2020; Khursheed et al., 2022).

5. Conclusion

In this work we evaluated the field efficacy of alternative commercial products and their mixtures to control preharvest citrus anthracnose caused by *Colletotrichum* spp., and ABS caused by *A. alternata*, on citrus fruits, as potential substitute to conventional Cu-based compounds. Field trials has been performed in different Sicilian citrus orchards to strengthen the reliability of our results in real disease scenarios. The results affirmed that all the alternative treatments, even under different disease pressure, were effective in reducing disease incidence and severity when compared to untreated control, and, in most of the trials, their efficacy was comparable or better than Cu-based treatments. Results were confirmed on different citrus species (*C. limon* and *C. sinensis*) and accessions ('Femminello Siracusano 2 KR', 'Tarocco Tapi' and 'Tarocco Sciré') with variable susceptibility to the target diseases, and on different pedoclimatic environments. However, climate parameters, plant host susceptibility and timing application are important factors in the development and management of fungal diseases. At this regard, the development and validation of predictive models that can estimate the real risk of infection and identify the optimal time for treatments according to the phenological stages of crop and climatic conditions encountered in the field might be particularly useful.

Concluding, the good efficacy of alternative products and their economic sustainability strongly encourages their further evaluations in natural conditions so to foster their application by growers in order to replace and/or reducing the use of Cu-based antimicrobials in integrated and organic citriculture in the near future.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix. ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2023.106520.

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