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ORIGINAL ARTICLE

Biological control of strawberry diseases by *Aureobasidium pullulans* and sugar beet extract under field conditions

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Abstract

Grey mould (caused by *Botrytis cinerea*) is the most important pathogen underlying high fungicide dependence in strawberry fields. Reliable biocontrol agents (BCAs) with improved efficiency are needed to replace fungicides. The yeast-like beneficial fungus *Aureobasidium pullulans* (AP-SLU6) has previously exhibited great potential to combat grey mould in greenhouse environments. Here we report results from a two-year full-factorial field trial in a conventional strawberry field, in which we tested two different concentrations of *A. pullulans* (10^7 CFU/ml and 10^6 CFU/ml) and sugar beet extract (SBE). The results showed that all the field treatments reduced grey mould severity postharvest and increased shelf life of the harvested fruit in both years. The best effect was achieved using the highest conidial concentration of *A. pullulans*, which also resulted in 53% higher fruit production compared to the control treatment at the end of the season, indicating a plant-growth promoting effect of the BCA. These results reveal that spray applications of these novel BCAs contribute to reliable biocontrol of grey mould, leading to improvement of the shelf life of strawberry sales boxes. These findings suggest that *A. pullulans* and SBE can contribute to a shift from chemical fungicides to sustainable methods without compromising cropping security.

Keywords *Aureobasidium pullulans* · Biological control · *Botrytis cinerea* · Plant growth promotion · Shelf life · Sugar beet extract

Introduction

Grey mould, caused by the metropolitan pathogen *Botrytis cinerea* Pers (perfect stage *Botryotinia fuckeliana*) is the single most important factor causing yield loss and fungicide dependence in commercial strawberry production, but other pathogens [e.g., *Mycospharella fragariae* (Tul.) Lindau, which causes leaf spot disease] and pest insects (e.g., sawflies, which physically damage leaves) also contribute substantially to damage (Delhomez et al. 1995; Petrasch et al. 2019; Wendin et al. 2019). As chemical plant protection products are gradually phased out, there is a need for new and improved biological control agents to combat these pathogens and pests (Stenberg et al. 2021).

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The use of some beneficial microbes, including *Bacillus amyloliquefaciens*, *B. subtilis*, and *Clonostachys rosea* (previously *Gliocladium catenulatum*), has been developed to combat grey mould, albeit with varying success (Samaras et al. 2021; Meng et al. 2022). Leaf spot disease and sawflies, on the other hand, are typically not the main targets of existing biocontrol products, although they sometimes show sensitivity to broad-spectrum products. Clearly, there is a need for additional and more efficient BCAs (Khan and Javaid 2022).

The yeast-like fungus *Aureobasidium pullulans* (De Bary) G. Armaud is one of a few candidate BCAs that are sufficiently effective to replace chemical fungicides (Di Francesco et al. 2020; Iqbal et al. 2021, 2022; Di Francesco et al. 2023). Several previous studies have shown that some strains effectively antagonize *B. cinerea* under greenhouse conditions (Di Francesco and Mari 2014; Di Francesco et al. 2020; Iqbal et al. 2021, 2022). The present study aimed to test whether equally promising results can be obtained when spray-applied to flowering plants in open fields. Although grey mould (*B. cinerea*) is the focal disease of this study, we also screen effects on common leaf spot disease and sawfly chewing damage. These other pathogens/pests were included because various strains of *A. pullulans* have previously showed a wide range of direct and indirect (via plant induction) mechanisms which may lead to broad-spectrum biocontrol. In addition, as Di Francesco et al. (2020) showed that *A. pullulans* can act as a plant-growth promotor for legumes, this current study was also designed to test whether it can increase the production of strawberry fruits.

In recent years, there are many reports of successful management of pathogens and diseases by using botanicals in different forms such as crude plant extracts (Banaras et al. 2021), purified compounds (Javed et al. 2021), and by using plant materials as soil amendments (Jabeen et al. 2021). In addition to *A. pullulans*, this study also includes treatments with sugar beet extract (SBE). One bi-product from sugar refining is lime that contains bound polyphenols and other impurities from the plant, representing a cheap raw material. We have previously used this material to make an ethanol-extract in order to induce plant defence in potato (Moushib et al. 2013). So far, the only proven effect has been a reduction of late blight (*Phytophthora infestans*) under controlled conditions. Induction of Pathogenesis Related Protein (PR1) was demonstrated and there was no direct toxic effect. After extraction, the remaining raw material can still be used as a soil amendment, as usual. This study aimed to test whether weekly application of *A. pullulans* (AP-SLU6) and SBE during flowering would lead to reduce the grey mould problem post-harvest and increase strawberry yield and shelf life.

Materials and methods

A. pullulans maintenance and formulation preparation

The *A. pullulans* strain AP-SLU6 was isolated from SLU campus Alnarp (Iqbal et al. 2021) and maintained on potato dextrose agar (PDA) medium (Oxoid; Basingstoke, Hampshire, England) at 25 °C for two weeks under dark conditions. It was revived from stock culture preserved in 20% (wt/vol) glycerol at – 80 °C. The conidia produced by the *A. pullulans* were harvested by adding 5–7 ml of sterile water to the fungal culture, then the surface of the mycelium was scraped with a spreader (Iqbal et al. 2021, 2022). One high and one low conidial concentration (10^7 and 10^6 CFU/ml, respectively) were used in the field experiment (described below) and these concentrations were determined using a haemocytometer (Hausser Scientific, Horsham, PA) under a light microscope (Laborlux12 Leitz, Germany).

Sugar Beet Extract (SBE) preparation and storage

The sugar beet extract (SBE) in this study was produced as described previously by Moushib et al. (2013). Briefly, SBE was obtained by adding 0.5 l 99.9% ethanol to each kg of the processed sugar beet biomass (lime), which had been stored at 8 °C until use. The mixture was thoroughly blended and left for 2 h to settle. The supernatant was filtered through two layers of cheesecloth and the filtrate was then centrifuged (3,000 rpm, 3 min at 5 °C). After this, the supernatant was stored at 8 °C until use. A 20% solution diluted with tap water was used for the experiments.

Field design and biocontrol treatments

The field experiment reported in this paper was carried out in a conventional commercial strawberry (*Fragaria × ananassa*) field in southern Sweden (geographic coordinates: 55°44’12.5"N 13°03’35.2"E). The field was established in 2019 using cultivar Florence. The plants were grown for two years prior to use in this study.

Four treatments were applied: (i) *A. pullulans* (10^7 CFU/ml), (ii) *A. pullulans* (10^6 CFU/ml), (iii) SBE (20%), and (iv) a control treatment where the plants were sprayed with tap water. The four treatments were applied in ten blocks, each consisting of a 10-metre-long double row. Each block was divided into four plots (each 2.5 m long with the double row, i.e., 80 cm wide), and each plot was randomly assigned to one treatment, such that all treatments were represented in each block. Each 2.5-metre plot was treated as an independent replicate. No disease symptoms were visible on the plants when the treatments started.

High or low *A. pullulans* conidial concentrations (10^7 and 10^6 CFU/ml, respectively) were sprayed all over the treated plants (above-ground parts) using electric backpack sprayers (Hardi BPE18). Similarly, 20% SBE was sprayed all over the plants in the SBE treatment and only water was sprayed on plants in the control plots. Tween 20 (0.1%) was added to all the prepared formulations just before spraying to plants.

The prepared formulations of *A. pullulans* and SBE were sprayed on all above-ground parts of the plants (including flowers, fruits and leaves) once every seven days for the seven weeks of the experiment (total six applications). During each application, a 20 l dose of each formulated treatment was sprayed onto each plot. The first application of the respective treatment was at the onset of the flowering and the last application was the week before the harvest period started.

Effects of *A. pullulans* and SBE on fruit shelf life

Over two years (2021–2022), the ripe fruit were harvested weekly, and to determine the effects of *A. pullulans* and SBE applications on the shelf life of harvested strawberries, we stored the sales boxes with fruit in at 4 °C for three weeks. Hokkanen et al. (2008) developed a protocol to measure the biocontrol efficiency of fruits in a sales box test by counting the number of days until the first grey mould symptom emerged. This approach is more relevant and useful from an applied perspective as strawberry sales boxes become unmarketable as soon as disease symptoms are visible. The effect of different treatments on shelf life was evaluated by scoring the emergence of grey mould symptoms every day during the incubation period of three weeks, following the disease scale described in previous papers (Adikaram et al. 2002; Iqbal et al. 2021). In short, four scores were used: 0 = no fungal growth, 1 = fungal growth only on the margin of any lesions, 2 = even but slight fungal growth all over the fruit, and 3 = dense fungal growth all over the fruit. The number of days it took for the fruits to show disease score 1 was used as a measure of shelf life.

Effects of *A. pullulans* and SBE on common leaf spot disease

The effect of *A. pullulans* and SBE on common leaf spot disease (*Mycospharella fragariae*) was determined by considering disease incidence and symptoms on the strawberry leaves, which had developed naturally under field conditions. To score leaf disease symptoms, we sampled five fully developed leaf samples from each plot. The leaves were selected by laying a pre-marked measuring stick (marked every 50 cm) along each plot – the top leaf closest to the five marks were sampled. All sampled leaves were digitized (scanned) using an image scanner and then visually scored on an electronic visual display. A modified disease severity index was used based on the visual estimation of fungal symptoms as described in the literature (Saharan and Mehta 2008). In short, score 0 = no fungal growth, 1 = 1–10% covered with fungal growth, 2 = 11–25% covered, 3 = 26–50% covered and 4 = > 50% leaf area covered with dense fungal growth.

Effect of *A. pullulans* and SBE on leaf damage in strawberry

The infestation of leaves and plant health were assessed by estimating the effect of *A. pullulans* and SBE on leaf herbivory by sawflies. We randomly picked five fully developed leaves from the uppermost part of each subplot and digitized them as described above (previous paragraph). Most of the damage was caused by sawfly larvae, *Cladius difformis* (Panzer). However, as sawfly numbers do not translate perfectly to leaf damage (which is more relevant from a crop protection point of view) we decided to score leaf damage rather than sawfly numbers for the purpose of this study, following the protocol of Koski et al. (2021). Thus, the chewing damage caused by sawfly larvae was assessed visually (by eye) as percentage leaf area consumed. We used an online training tool (ZAX Herbivory Trainer; www.zaxherbivorytrainer.com) to make sure that the visual estimates were as accurate as possible.

Effect of *A. pullulans* and SBE on fruit production (plant growth promotion)

During the first year of the experiment (2021), we measured the effects of *A. pullulans* and SBE on the total strawberry production (kg/plot) in comparison to the control plots. All ripe fruit from all plots were harvested and weighed in the laboratory for the purpose of this study.

Statistical analyses

All data for two consecutive years (2021–2022) were analysed using analysis of variance (ANOVA) with a Fit Mixed Effects Model implemented in Minitab Statistical Software version 18.1 (Minitab Inc., State College, PA, USA). Treatment, Block, Year, and Picking date (nested under Year) were used as explanatory factors, while strawberry shelf life (days until grey mould symptoms were visible in sales boxes), yield (kg/plot), common leaf spot infection rate (score 0–4), and leaf herbivory (%), respectively, were used as dependent variables. Subsequent pairwise comparisons were carried out using Fisher's least significant difference at a 95% significance level.

Results

Effects of *A. pullulans* and SBE on fruit shelf life

Fit mixed effect analysis of variance was performed to determine the effect of *A. pullulans* and SBE on fruit shelf life, with data collected over two years. The results of ANOVA showed that differences between the years were not significant ($P = 0.568$), whereas differences between the treatments were highly significant ($P \leq 0.005$) as shown in Fig. 1 (Table S1). The sales boxes of strawberries that had developed from flowers treated with *A. pullulans* either with high or low concentration or with SBE showed a significantly ($P \leq 0.005$) prolonged shelf life compared with the control treatment over different weeks in both years (Fig. 1A and

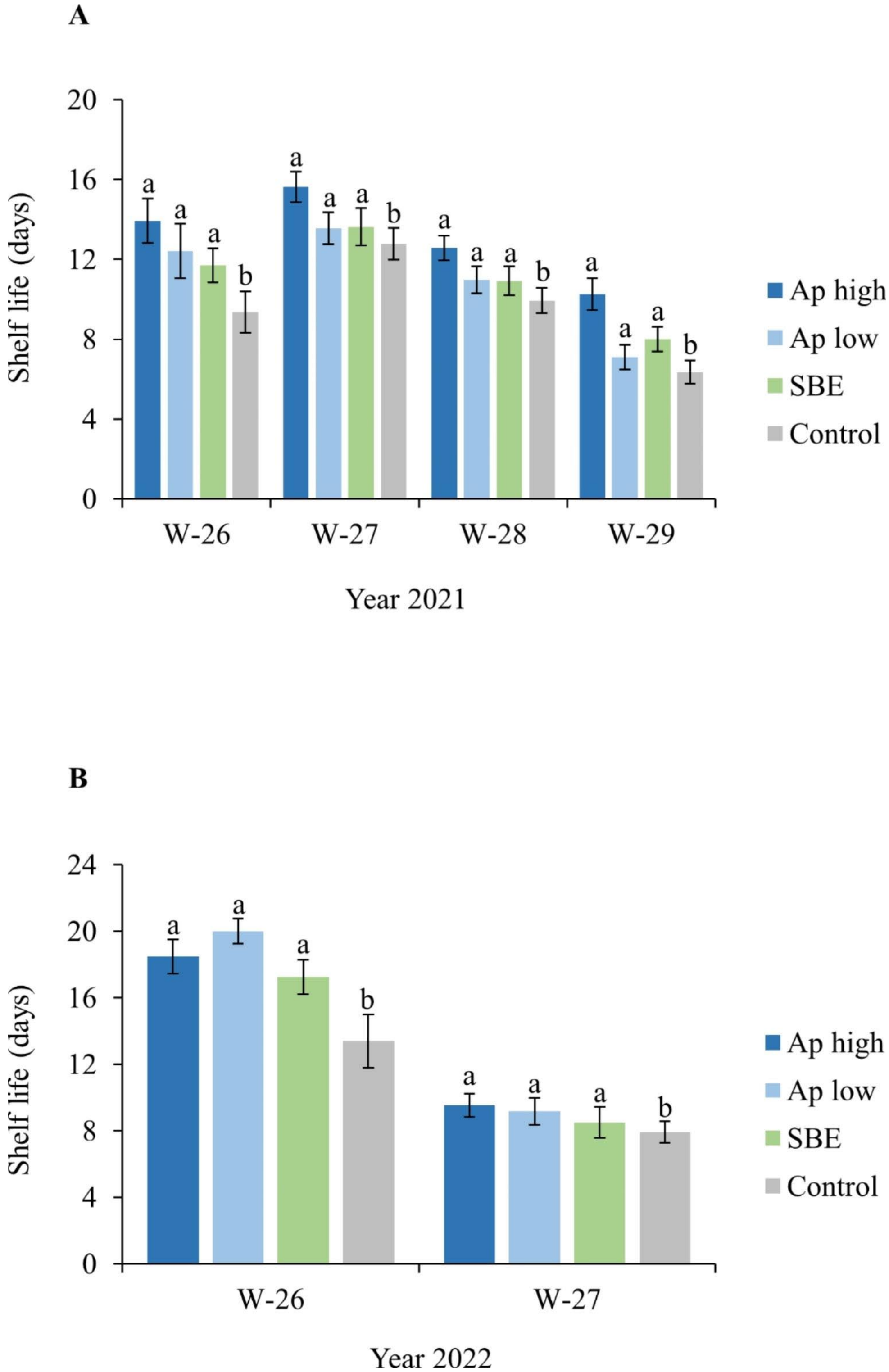

Fig. 1 Effect of *Aureobasidium pullulans* (AP-SLU6, high and low conidial concentrations) and sugar beet extract (SBE) on the shelf life of harvested strawberry fruit incubated at 4 °C in plastic sales boxes. **A**; Fruit collected during four weeks in 2021, and **B**; fruit collected during two weeks in 2022. Grey mould disease severity was recorded every day for a period of 3-weeks for each sales box. Error bars indicate standard errors based on 10 biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) as determined by Tukey-Kramer tests. Statistically significant differences between treatments and years are provided in Table S1

B). However, the effect of a higher concentration of *A. pullulans* had a significantly more pronounced effect on shelf life (36% longer shelf life) followed by *A. pullulans* lower concentration (24%) as compared with the control treatment. In contrast, SBE increased the shelf life by 16% compared with the control treatment (Fig. 1A and B). As expected, no significant ($P = 0.178$) difference was found between the years and different treatment combinations (Fig. 1).

Effects of *A. pullulans* and SBE on common leaf spot disease

Plots sprayed with *A. pullulans* and SBE showed lower levels of common leaf spot disease, but this reduction was not statistically significant ($P > 0.05$). However, a significant difference ($P \leq 0.005$) was found between the years with regard to disease severity (Fig. 2; Table S2). In 2021, *A. pullulans* application reduced disease severity by approximately 32% compared with the control treatment. In contrast, SBE reduced disease severity by only 21% compared with the control treatment (Fig. 2A). In 2022, only *A.*

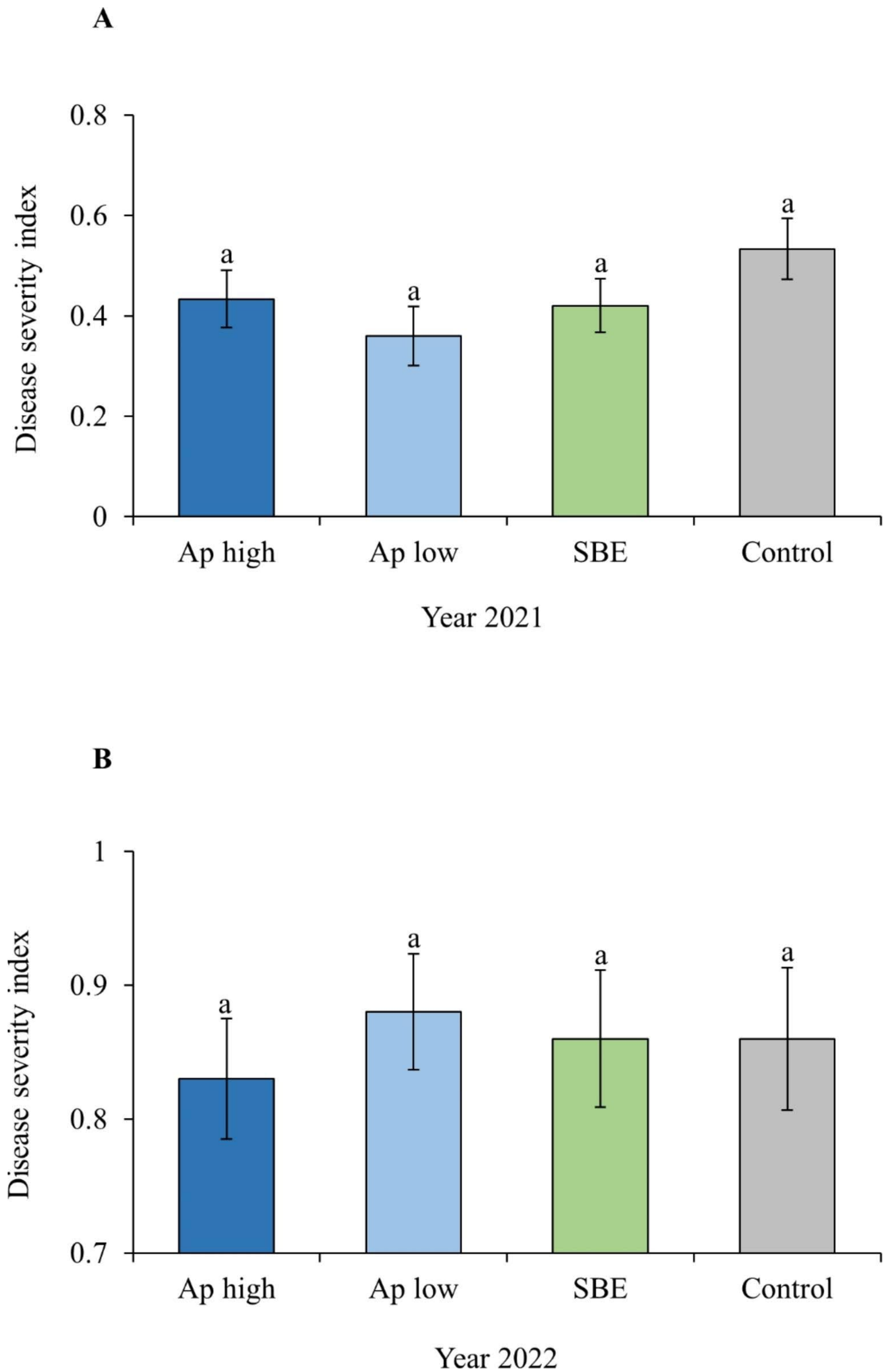

Fig. 2 Effect of *Aureobasidium pullulans* (AP-SLU6, high and low conidial concentrations) and sugar beet extract (SBE) against *Mycospharella fragariae* which causes common leaf spot disease of strawberry leaves. **A**; year 2021, and **B**; year 2022. Error bars indicate standard errors based on 10 biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) as determined by Tukey-Kramer tests. No significant effects of the treatments were found on disease as shown in Table S2

pullulans reduced disease severity, and this by approximately 4% compared with the control treatment (Fig. 2B).

Effect of *A. pullulans* and SBE on leaf damage

The effect of *A. pullulans* and SBE on chewing damage to leaves was evaluated over two years. The results reveal a significant difference between the years ($P \leq 0.005$), indicating that herbivory changed over time (Fig. 3; Table S3). However, although the damage was lower in plots sprayed with *A. pullulans*, this reduction was not statistically significant ($P > 0.05$). In 2021, *A. pullulans* application reduced leaf damage by approximately 5% compared with the control treatment, however, interestingly SBE enhanced

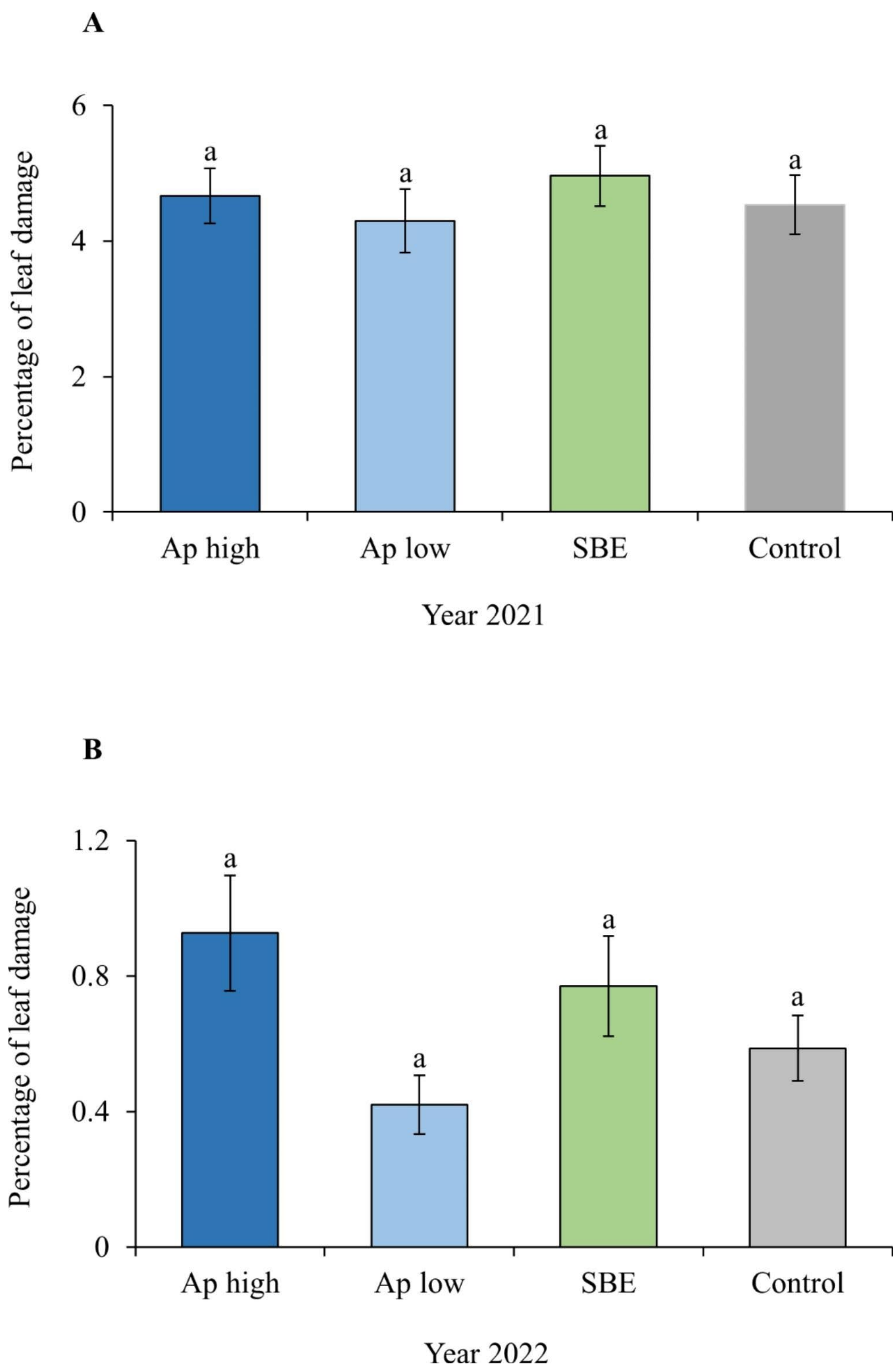

Fig. 3 Effect of *Aureobasidium pullulans* (AP-SLU6, high and low conidial concentrations) and sugar beet extract (SBE) on the leaf chewing damage caused by sawflies to strawberry leaves. **A**; year 2021, and **B**; year 2022. Error bars indicate standard errors based on 10 biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) as determined by Tukey-Kramer tests. No significant effects of the treatments were found on leaf damage as represented in Table S3

leaf damage (9%) compared with the control treatment (Fig. 3A). Similarly, in 2022, the lower conidial concentration of *A. pullulans* reduced the damaged area by about 28% compared with the control treatment, while the higher conidial concentration and SBE application increased the damaged area by approximately 57% and 31%, respectively (Fig. 3B).

Effect of *A. pullulans* and SBE on fruit production

The effects of *A. pullulans* and SBE on fruit production were evaluated in 2021 only. Fruit production in weeks 26, 27 and 28 were not significantly affected by any treatment ($P > 0.05$). However, fruit production at the end of the season (week 29) was strong (53%) and significantly ($P \leq 0.018$) higher for plots sprayed with the high conidial concentration of *A. pullulans* (Fig. 4; Table S4). The other treatments were not significantly different from the control (Fig. 4).

Discussion

The results from this study show that spray applications of *A. pullulans* and SBE during flowering can reduce grey mould disease severity and prolong the shelf life of strawberries. Previous trials have shown that application of *A. pullulans* in greenhouse environments can increase the shelf life of strawberries (Iqbal et al. 2021, 2022), but the present study is the first one showing that the positive effects are equally strong outdoors in conventional commercial strawberry fields. Previously, it has been shown that *A. pullulans* can control the rotting of other crops, including grapes, kiwi fruit, cherries, and tomato (Ippolito et al. 1997; Schena et al. 1999; Di Francesco et al. 2020, 2023). The improved shelf life found in this study is important because food loss due to postharvest grey mould development in sales boxes is a very big economic and ecological problem (Shafiee-Jood and Cai 2016), and even minor improvements in shelf life can reduce food loss and increase revenue considerably according to retailers in Sweden.

The results of the current study further showed that SBE also reduced grey mould infection in fruit and thereby increased shelf life. Previously, it has been shown that SBE can assist in reducing late potato blight caused by *P. infestans* by inducing resistance without any direct toxic effects (Moushib et al. 2013). The underlying mechanism behind reduced grey mould symptoms demonstrated in the current study is not known. A major advantage of SBE is its low price, and high production capacity, which would make it readily available to many farmers.

Besides the capacity of *A. pullulans* to combat grey mould, this study also shows that the highest conidial concentration of *A. pullulans* increased fruit production by more than 50% at the end of the season. This increase in production suggests that *A. pullulans* may act as a growth promotor for strawberry. Di Francesco et al. (2021) have shown that other strains of *A. pullulans* can stimulate the growth of legumes. Although the effect on total yield in this study was small (as the effect only appeared at the end of the season), the capacity of *A. pullulans* to stimulate the growth of strawberry is very interesting and should be investigated further in future studies. It is likely that maximal effects of plant growth promotion can only be achieved if *A. pullulans* is applied early in the growing period – in this experiment there was no application before flowering.

Although *A. pullulans* typically has multiple modes of action, we did not find any significant effects of our treatments on common leaf spot symptoms or chewing damage

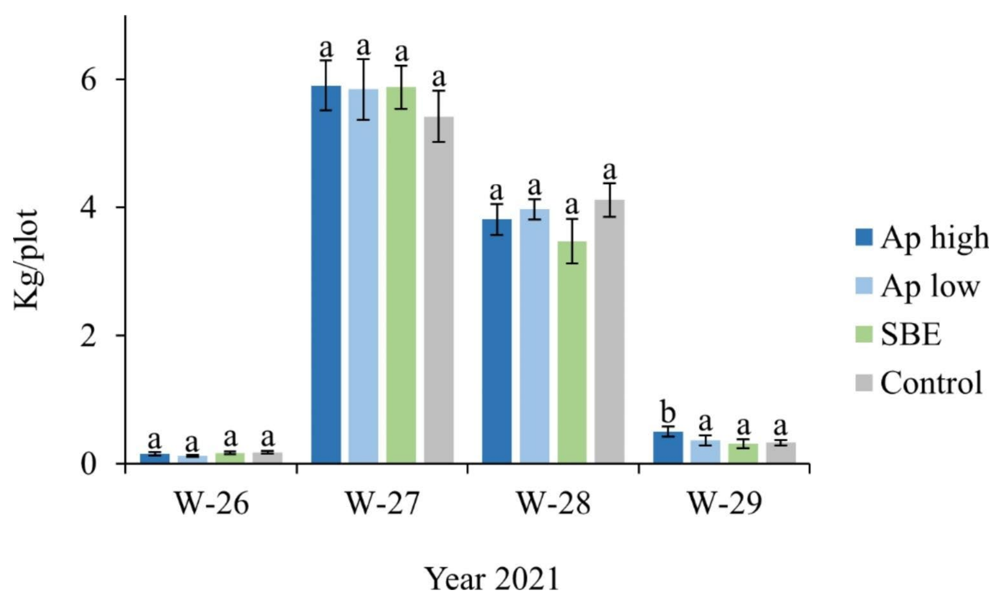

Fig. 4 Effect of *Aureobasidium pullulans* (AP-SLU6, high and low conidial concentrations) and sugar beet extract (SBE) on the total yield (Kg/plot) of strawberries. Error bars indicate standard errors based on 10 biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) as determined by Tukey-Kramer tests

caused by sawflies. Previous research indicated that *A. pullulans* can reduce crown rot and root rot disease, in addition to grey mould, thus suggesting a broader spectrum of targeted pathogens (Iqbal et al. 2021). Symptoms of these latter diseases were not found in our plantation, and the lack of effects on common leaf spot disease suggests that grey mould remains the main target of *A. pullulans* under Scandinavian field conditions.

The strong antagonistic effect of *A. pullulans* against grey mould has now been shown under several conditions and with several application methods (e.g., spraying and bee vectoring) (Iqbal et al. 2021, 2022, this study). An important next step is to identify the most promising modes of action and to optimize them further. However, given the strong disease reduction post-harvest and the shelf-life increase, it seems clear that even the current strain would confer important gains on various strawberry production systems.

Overall, this study reports the first results of field application of *A. pullulans* (AP-SLU) and SBE in conventional strawberry fields and demonstrates that weekly spray applications during flowering reduced grey mould development post-harvest and increased shelf life. The development of *A. pullulans* as a reliable and efficient BCA could be an important step to catalyse a shift from chemical fungicides to sustainable biocontrol for strawberry (Di Francesco et al. 2023). Given the current heavy dependence on fungicides, such a development would be an important contribution to reach the European Commission's Green Deal goals for 2030.

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Author contributions MI, EA and JAS conceived and designed the experiments. MI performed the experiments and analysed the data. MI wrote the manuscript with critical input from EA and JAS. All authors read and approved the manuscript.

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Data Availability All data generated or analysed during this study are included in this manuscript.

Code Availability Not applicable.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors consent to publication.

Conflicts of interest The authors declare that that they have no conflict of interest related to funding or otherwise.

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