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Native *Bacillus subtilis* Strains Efficiently Control Lupin Anthracnose Both under Greenhouse and in Field Conditions

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Abstract*— *Colletotrichum acutatum* is the cause of lupin anthracnose in the Andean zone. The pathogen affects the crop throughout its entire production cycle, causing losses of up to 100%. In previous studies, native strains of *B. subtilis* (CtpxS1-1; CtpxS2-1 and CtpxZ3) from the province of Cotopaxi reduced anthracnose infections in seed and seedlings. This study evaluated the potential biological control of these strains by using two lupin anthracnose susceptible cultivars under greenhouse conditions and in the field. Plants of I-451 Güaranguito and I-450 Andino cultivars were treated with active biomass from each *B. subtilis* strain. Pre-inoculated plants that received a bacterial concentration of 1×10^9 colony-forming units per milliliter (CFU/ml) showed a reduction by ten times in the stem lesion diameter when compared with plants artificially infected with the pathogen alone in greenhouse evaluations. Sequential applications of antagonists every two-week allow for quantifying their biocontrol efficiency under field conditions. A significant ($P<0.05$) reduction was found for the area under the disease progression curve (AUDPC) when comparing the treatments that received *B. subtilis* with the control plants naturally infected with anthracnose, in both lupin susceptible cultivars, along the 2015 and 2019 growing seasons. Analysis of population dynamics in the phyllosphere of lupin showed that *B. subtilis* survived over 7.0 LOG CFU/g on lupin leaf and stem surface throughout four evaluations. This fact was associated with its protective effect along vegetative, flowering, and pod-filling phenological stages. Results of this study showed that native *B. subtilis* strains efficiently control lupin anthracnose.

***Keywords*—Lupin anthracnose; *Bacillus subtilis*; *Colletotrichum acutatum*; biological control; AUDPC.**

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I. INTRODUCTION

Lupin (*Lupinus mutabilis* Sweet) tarwi, chocho or Andean lupin is a grain legume crop that originated in the Andean area of South America, cultivated since pre-Columbian times, with a growing demand due to its high nutritional value [1]. This legume is characterized by its adaptation to sandy soils, is poor in nutrients, and requires only 600 mm of water throughout its entire growing cycle [2]. Currently, at the national and regional level, its uses have been diversified in different agricultural and industrial fields, especially for the preparation of food, medicine, forage, and rituals of indigenous cultures, standing out among other grains for their great nutritional value [2]. Because lupin has high levels of protein (45-50%), higher than soybeans [3], there is currently great interest in its cultivation and industrialization at a global level [2].

Anthracnose is considered the world's most devastating lupin disease due to its rapid spread, affecting the plant in all its phenological stages, and causing losses that can reach 100% [4], [5]. In the Andean zone the cause of anthracnose is *Colletotrichum acutatum* [6]. The infected seed is the source of primary inoculum and the cause of subsequent epidemics in the field especially when there are continuous rains [1], [6]. The pathogen survives underneath and in the seed coat, producing a dark shared inter and intracellular mycelium [7]. During seed germination, the fungal pathogen moves from the seed coat to the emerged seedlings. Then, mycelia initially colonize the cotyledons, then the radicle and plumule [8]. The typical bending of the central stem is the first symptom, easily visible before flowering, but circular and elongated lesions can also be observed on stems, pods, and infected seeds with salmon-pink masses [4], [6]. Finally, production is severely affected due to infections caused by anthracnose in the adult plant, pods, and seed [6], [8].

The conventional management of anthracnose is done through seed disinfection with systemic fungicides to reduce transmission of the pathogen to the seedling. However, no

fungicides eradicate the pathogen because they do not completely reach its survival site [9]. For this reason, the application of dry heat in convection ovens [10], ultraviolet-C radiation [3] UV-B solar radiation converted into thermal energy plus 50% of accumulated radiation in a solar oven for the disinfection of seed [11], and biological treatments such as *Bacillus subtilis* for reducing infection on seed [11] constitute sustainable alternatives to reduce anthracnose infections in seed without causing damage to the environment and human health, including animal health.

From a commercial point of view, the use of biopesticides based on *B. subtilis* has been widely demonstrated efficiency for controlling multiple fields and postharvest fungal pathogens. Foliar applications of *Bacillus* sp. HA1 strain efficiently controlled the tomato mosaic virus [12]. Other local experiences sustain that applications of native *B. subtilis* to cacao phylloplane have proven efficiency in the biocontrol of cacao diseases, significantly reducing the incidence compared to the untreated control [13]. Many other studies show that various species of *Bacillus* spp, including *B. subtilis* use several ways to prevent disease infection or reduce disease severity in various crops [12], [14]–[16]and additionally induce positive changes in the growth and resistance of inoculated plants [3], [15], [19].

Due to its efficiency in reducing anthracnose caused by *C. acutatum* with latent infections in lupin seed and seedlings in previous studies [15], [16] here we do a *bioprospection* of native *B. subtilis* to find out their potential response under control conditions and in the field on normal lupin production conditions in the town of El Chaupi, province of Pichincha, Republic of Ecuador.

II. Materials and Methods

Seed of the anthracnose susceptible cultivars INIAP-450 Andino and INIAP-451 Guaranguito were used for both greenhouse and field trails experiments [10], [16]. The research flow chart is represented in Fig. 1.

A. B. subtilis Strains and Culture Conditions

B. subtilis strains CtpxS2-1, CtpxS1-1 and CtpxZ3 collected from lupin pods phylloplane and formed lupin seed that efficiently reduced incidence of *C. acutatum* in seed [16] and seedlings [15] were used in this study. Criobilles AEB 400100 were used to keep strains at −80°C for long-term storage at the Laboratorio de Control Biológico, Universidad de las Fuerzas Armadas ESPE, Sangolqui - Ecuador. Before each experiment, a new bacterial culture was obtained from frozen stocks and grown at 30°C, 150 rpm for 72 h in the optimal medium MOLP for lipopeptide production [17]. From this culture in MOLP of 72-hour-old were prepared living cells suspensions of each *B. subtilis* strain at a concentration of 10^9 CFU/ ml [3].

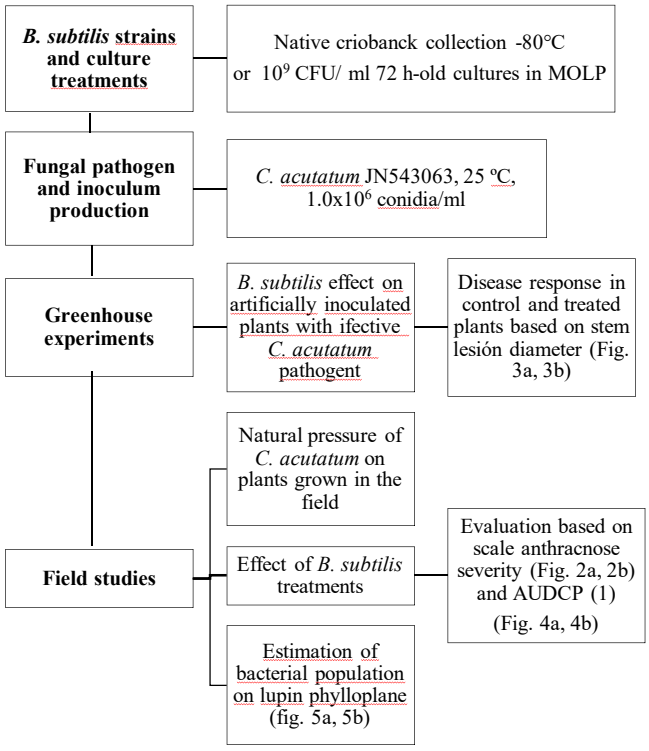

Fig. 1 Research flow native *Bacillus subtilis* strains efficiently control lupin anthracnose both under greenhouse and in-field conditions

B. Fungal pathogen and production of inoculum

The fungal pathogen used for greenhouse studies was Lup 18 registered at EMBL as accession number JN543063. The strain of *C. acutatum* belongs to the culture collection of Laboratorio de Fitopatología, Universidad de las Fuerzas Armadas ESPE, Ecuador. Pods and seeds of lupin crops which showed symptoms caused by natural infections with anthracnose and grown in the province of Cotopaxi – Ecuador was collected to isolate the pathogenic fungus as described by Falconí, Visser, and van Heusden [6].

The fungal pathogen was routinely cultured and incubated as described by Falconí, Visser, and van Heusden [6]. To produce conidia from artificial infections on plant, the pathogen was periodically inoculated to one-month old healthy seedlings. The pathogenic fungus strain was kept in tubes with slanted potato dextrose agar and was subcultured in Petri dishes with PDA plus chloramphenicol (500 mg/l). The strain was incubated for 10 days at 25 ± 1ºC. To prepare conidia suspensions, fungal surface growth was flooded with a sterile solution of NaCl 0.8% + Tween-80 0.1% and scraped mycelia with a sterilized metal. A hemocytometer helped determine spore concentration that was adjusted to 1.0 x 10^6 conidia/ml [10].

C. Greenhouse Experiments, Plant Material and Experimental Design

The experiments were performed in a greenhouse at the Carrera de Ingenieria Agropecuaria, Departamento de Ciencias de la Vida, Universidad de las Fuerzas Armadas ESPE, Ecuador (0° 23” 20” S, 78° 24” 44” W, 2748 m.a.s.l). Seeds that did not show anthracnose infections were selected and sterilized superficially with 0.5% sodium hypochlorite for 5 min and rinsed with sterile water. The substrate for lupin

plant growth was a mixture in equal parts of coconut fiber, sterilized soil, and pumice. Plastic pots of 4 kg capacity were filled with this mixture, and five seeds were sown in each one. Three seedlings of equal size were left per experimental unit to homogenize the development of the plants, 15 days after sowing. Plants were grown in a greenhouse at 12 ± 2ºC night / 20 ± 2ºC day of temperature, photoperiod of 12 h, and relative humidity of 70 ± 10% [10]. The experiment was conducted in a completely randomized design: three *B. subtilis* strains and one non-inoculated treatment control, with four repetitions.

D. Evaluation of B. subtilis Strains on Lupin Anthracnose in Pots Under Greenhouse Conditions

A wound was artificially made with a hypodermic syringe at the early vegetative stage of six-week-old lupin plants' main apical stem. The same depth was made for each wound. Plants had 6 or 7 leaves– internodes, internodes elongate on main stem, but cotyledons were absent. Inoculations were conducted according to the methods practiced by Falconi,Visser, and Van Heusden [18] with modifications. By using a micropipette, 25 µl of bacterial suspension was injected in each wound, and after around 5 min, 25 µl of *C. acutatum* spore suspension was inoculated in the same way as the antagonist. Small black plastic bags were used to cover the inoculated plant completely, and small pieces of cotton were soaked in sterile distilled water to stimulate infection. After 72 h, the bags were removed [18]. The level of infection was compared with that of the stems inoculated with the pathogen alone.

To evaluate the effect of *B. subtilis* strains on disease response, stem lesion diameter was recorded using a caliper after 7, 14, 21, and 28 days of inoculation, and the amount of anthracnose was measured on individual plants by using the severity scale described in Figure 2.

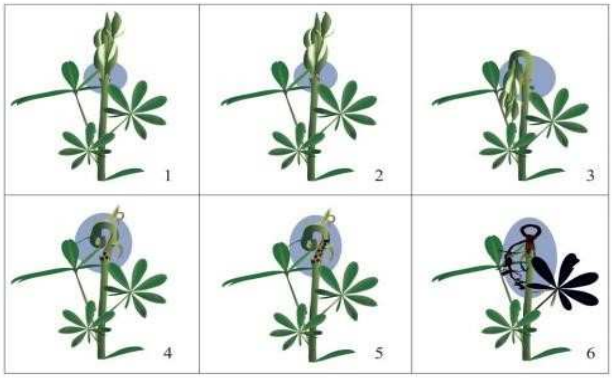

Fig. 2 Anthracnose severity was based on a 1-6 scale: 1 = no symptoms or healthy plant, 2 = lesion lest than 2 mm at the point of injury, sporulation absent, 3= lesions of 2-5 mm main stem twisted, little sporulation, 4 = lesions of 5-10 mm size at the point wound was made, little sporulation, 5 = presence of lesions (from 10 to up 20 mm) on stem, in conjunction with necrotic tissue, abundant sporulation, 6 = dead plant or necrotic plant, severely affected [21].

E. Biological Control Trials in the Field, Plant Material, Experimental Design, and Experimental Management

Fieldwork was conducted in the Province of Pichincha, Mejía County, Chaupi Parish (latitude 0.6018813 S, longitude -78.6420014, altitude 3242 m.a.s.l.), during 2015 and 2019 growing seasons, using the susceptible anthracnose cultivars I-450 Andino and I-451 Guaranguito. Lupin was cultivated during previous years in the field used for this study. Treatments were allocated in a Randomized Complete

Block Design with four replications. The 32 experimental plots were 5.0 x 4.0 meters, one-meter distance row to row, and Inter-row spacing of 0.3m, and three seeds of the corresponding cultivar of lupin sown in each hole. The experimental field was surrounded by a border of the two susceptible lupin cultivars as a physical barrier and a disease spreader.

Management of the experiment was according to Falconí [1]. The pesticide Thiodan (Endosulfan) 4 ml/l of tap water was applied at the time of sowing and every two weeks for up to three months to control insect pests. Doses at the rate of 175 kg/ha of N + P_2O_5 + K_2O were applied for soil fertility after 35 days of sowing (DAS); at that time, plants on the plots were also weeded with local hoeing instruments. As usual, at 55 and 90 DAS, the base of the stem of each plant was covered with the surrounding soil for root protection and for plant growth promotion. *Agrotis ypsilon* and other insects appeared between 60-90 days after planting. Thiodan (Endosulfan) 4 ml/l of water was used for control. Other symptoms caused by aerial diseases of lupin such as *Ascochyta* sp., *Uromyces* sp., *Ovularia* sp., and lupin rots caused by *Sclerotinia* sp. showed up along vegetative, flowering, pod-filling, and harvest stages. No fungicides were used for control. Biol (organic foliar fertilizer) 1ml/l of water was applied at blossom time and pod-filling seasons, according to Falconí [1].

F. Inoculations of B. Subtilis Strains on Lupin Phyllosphere

The treatments were active biomass from 72-h old bacterial cultures in MOLP, growth at a concentration of 10^9 CFU/ml of *B. subtilis* (CtpxS1-1; Ctpx-S2-1 and CtpxZ3). Plants to be treated were identified, with ribbons of different colors and 3 ml of the liquid suspension's doses were applied with a manual micro-sprayer in the apical area to cover meristems, leaves, and main stem until inoculum run-off. Sixteen plants were randomly selected in each experimental unit, 4 plants for each treatment plus 4 plants for the control inoculated with natural pathogen pressure. The first application was conducted 60 days after sowing (DAS), and subsequent applications were made with a frequency of 15 days on the following phenological stages: 75 DAS developmental stage, 90 DAS pre-flowering stage, 105 DAS flowering stage, and 120 DAS early pod-filling stage. For cell concentration assessment of *B. subtilis*, apical lupin leaves samples were collected before each inoculation following procedure of Yánez-Mendizábal et al.[19] with modifications.

G. Evaluation of B. subtilis Strains on Lupin Anthracnose in the Field

The 1-6 scale described in Fig. 2 was used to quantify the amount of anthracnose on individual plants and to quantify the effect of *B. subtilis* strains on disease response. The multiple evaluations of severity collected over time was used to calculate Area Under Disease Progress Curve (AUDPC) applying Shaner y Finney´s formula [20]:

$$ABCPE = \sum_{i=1}^{n-1} \left(\frac{yi+yi+1}{2}\right)(ti+1-ti) \qquad (1)$$

Where: "t" = each reading time, "y" = severity by sampling, "n" = number of readings, "t" = represent days after sowing.

F. Estimation of bacterial populations on the phylloplane of lupin

Ten grams of inoculated leaves, on designated plants, were randomly selected and placed in 90 ml of 5% PBS to determine the survival of *B. subtilis* strains on the phylloplane of lupin plant by an adaptation of the serial dilution-plating method [19], [21]. Each sample was shaken for 5 min at 100 rpm and from this serial dilution banks were prepared up to the 10^{-10} dilution. Aliquots of 20 µL were taken from each dilution and plated in triplicate using the surface seeding technique in NYDA medium. The samples were incubated at 28 ± 1°C for 24 hours, and colonies counted bacterial population were estimated as colony-forming units per gram. First application was 45 DAS, and the assessments were conducted fortnightly, before each spray, 60 days after sowing (DAS), at developmental stage 75-DAS, pre-flowering stage 90 DAS, flowering 105 DAS, and early pod-filling 120 DAS. To add homogeneity to the variance, data was transformed to $\log_{10}$ CFU/g. The percentage of bacteria surviving was calculated by using the formula:

$$Survival\ \% = Nf\ x\ 100\ /Ni \qquad (2)$$

Where Ni = CFU/g of plant in suspension before application, Nf = CFU/g of plant in each treatment time [19].

G. Statistical analysis

The experiments were repeated two times, and the data from distinct experiments was combined for statistical analysis. The figures were constructed with the plotted data where values are represented as means plus the standard deviation (± SD) of four repetitions per sample. Analysis was carried out using the Student Newman and Keuls (SNK) test ($P < 0.05$) following one-way analysis of variance (ANOVA) using Infostat software.

III. Results and Discussion

As a biocontrol agent, native *B. subtilis* strains have been previously reported to control lupin anthracnose on seed and seedlings [15], [16]. To evaluate the effect of these strains on the control of lupin anthracnose, a series of experiments was conducted in the greenhouse and the field.

A. Evaluation of B. subtilis Strains on Lupin Anthracnose in Pots under Greenhouse Conditions

The lesion diameter on lupin stems preinoculated with *B. subtilis* on artificially wounded stems at the greenhouse trials are shown in Fig. 3A, B. Treatments that received the *B. subtilis* strains did not significantly affect the mean diameter of lesion, but they were significant ($P < 0.05$) lower than the control inoculated with pathogen alone.

In our study, lupin stem wounds of treatments with *B. subtilis* CtpxZ3 before *C. acutatum* were numerically less effective, but significant ($P<0.05$) equal than the two other strains giving stem lesion diameter up to 0.29 mm^2 after 7 days and increasing to 0.38 mm^2 after 28 days of inoculation in I-450 Andino and from 0.29 to 0.34 in I-451 Guaranguito cultivars, respectively. However, significant ($P<0.05$) differences were observed among the four evaluations when native *B. subtilis* were applied compared with the control inoculated with pathogen alone that reached up to 2.0 cm^2 after 28 days of inoculation Figures 3a-3b. In lupin stems, lesions caused by anthracnose reach a maximum of 20 mm in diameter, are oval in shape and usually have a salmon color due to the spore masses of the pathogen [5], [8]. Numerous studies indicate the possible mechanisms of biological control of plant diseases. Mechanisms may be one or a combination of antibiosis, anticipating nutrient supplies also known as competition for nutrients, and competition for sites of infection between *B. subtilis* and the pathogen [22]. Previous studies confirmed that one mechanism for native *B. subtilis* strains to suppress lupin anthracnose is the production of lipopetides such as fengycin, iturrin and surfactin [16].

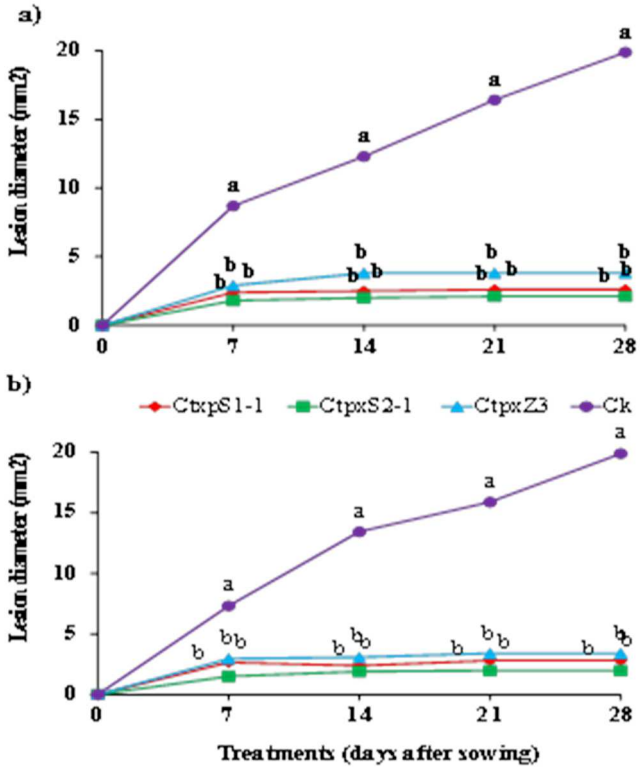

Fig. 3 Effect of *B. subtilis* Ctpx-S1; Ctpx-S2 and Ctpx-Z3 on the development of stem lesion on 6-week-old lupin (*L. mutabilis*) anthracnose susceptible cultivars a: I.450 Andino; b: I451 Guaranguito. Wounded sites were prior inoculations with 25 µl of *Bacillus* strains 10^9 CFU/ml following the application of 25 µl of *C. acutatum* spores' suspensions 1.0 x 10^6 conidia/ml. Disease assessment at 7, 14, 21 and 28 days after inoculation under greenhouse conditions. Ck: treatment inoculated with *C. acutatum* alone. Means with the same letter, for each evaluation date, are not significantly different according to SNK test ($P < 0.05$).

B. Evaluation of subtilis strains on lupin anthracnose in the field

The amount of anthracnose by effect of B. *subtilis* strains was the product of disease response on lupin plants in the field. Anthracnose was quantified based on the 1-6 scale (Fig. 2) and Area Under Disease Progress Curve calculated with severity data of multiple evaluations collected over time.

C. Reduction of area under disease progress curve (AUDPC) by effect of B. subtilis native strains

Figure 4 refers to the effect of native *B. subtilis* in reducing AUDPC in field trials under natural pathogen pressure. Data

indicate that all biological treatments significant (P<0.05) reduced AUDPC compared to control that showed large stem lesions, accompanied by necrotic tissue 90 DAS and abundant sporulation, and in some cases dead plant from 105 to 120 DAS. Lupin anthracnose can crack the stem at the site of infection; multiple lesions can lead to stem twisting or complete stem collapse [6]. The AUDPC was reduced with sequential applications of antagonists every two-week reaching up to 529 after 120 DAS, compared to the AUDPC of 1252 that reached the untreated control in the cultivar I-450 Andino (Fig. 4a). In general, the treatments with bacterial suspensions resulted in healthier lupin stems (pods, leaves, and other agronomic traits linked to production), that resembled other studies by using unmanned aerial vehicle imaging [25]. Previous studies on seeds and seedling show that antibiosis and the induction of plant resistance by the antagonist bacteria may cause disease control [3], [19]. For I-451 Guaranguito cultivar, *B. subtilis* strains also decreased AUDPC to 51% of *C. acutatum* compared to the control after four application sequences (Fig. 4B). Studies with *B. subtilis* decreased potato common scab severity from 80% in control to 34%, and 56% in control to 5%, respectively, when combined with vetch mulch (control: 98%), demonstrating that microbial response can be influenced by the cover crop [23].

The suppression of the disease by *B. subtilis* strains in the greenhouse was as marked as that observed in the field (Fig. 3a-3b; and Fig. 4a-4b, respectively). This suggests that native *B. subtilis* controlled a single pathogen strain of *C. acutatum* on artificial infections as well as possibly several races of the pathogen from natural infections in the field. Studies in other pathosystems show that cell degradation of conidia and hyphae of *Fusarium oxysporum* cause of bulb rot disease of *Fritillaria taipaiensis* P.Y. Li. was observed when treated with *B. subtilis* [24], results that are in line with our findings where extract of lipopeptides of native *B. subtilis* prevented *C. acutatum* infections by inhibiting conidia and mycelium and that the production of lipopeptides similar to fengycine by activation of genes C and E of fengycin has a direct antifungal effect [16]. Synthetic chemical fungicides could be substituted for the antimicrobial metabolites produced by *B. subtilis*, or in turn bacterial metabolites could be used as a supplement to fungicides to control plant diseases [8], [25].

The significant reduction (P<0.05) of AUDPC by *B. subtilis* CtpxS2-1 (335, 384), CtpxS1-1 (426, 455), and CtpxZ3 (528, 489) compared to controls (1252, 1010) in I-450 Andino and I-451 Guaranguito cultivars, respectively (Fig. 4a-4b), may be due to *B. subtilis* have a cascade effect on the different components of the disease triangle, that is, on the environment, the host plant or the pathogen; therefore it can affect the onset and progress of the disease and also turn on resistance genes in the host [24]. Some studies show that *B. subtilis* induces disease resistance in the plant or promotes plant growth, making it easier for plants to control pathogenic infections [27]. In addition, it can use diverse mechanisms, including improving nutrient availability, altering phytohormone homeostasis, and producing antimicrobials and triggering induced systemic resistance [26].

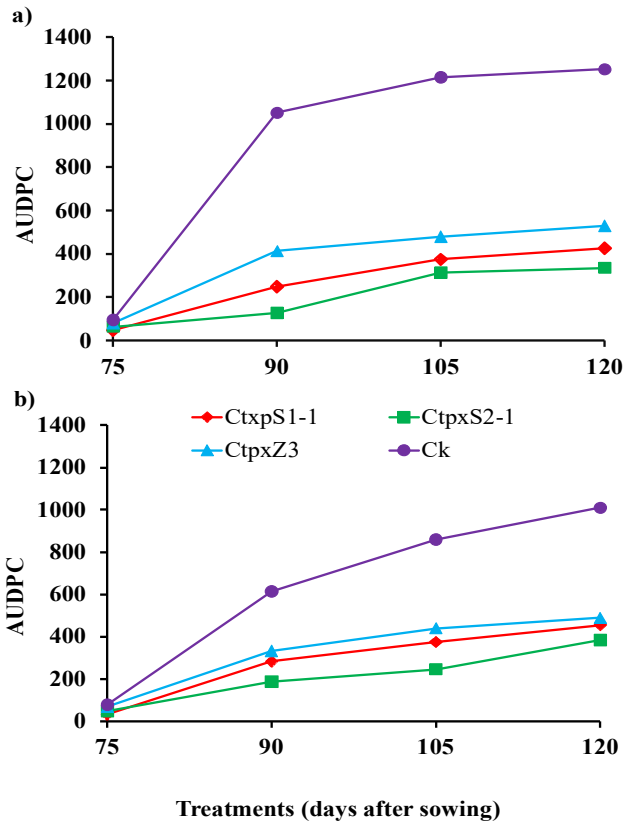

Fig. 4 The Area under disease progress curve (AUDPC) of anthracnose caused by *C. acutatum* on two lupin susceptible cultivars (a: I.450 Andino; b: I-451 Garnaut) (under natural pressure of inoculum is represented as the control (Ck) and the AUDPC by effect of *B. subtilis* CtpxS1; Ctpx-S2and Ctpx-Z3. AUDPC was calculated based on scores for lupin anthracnose severity based on the 1-to-6 scale [20] described in methods. Data from the 2015 and 2019 growing seasons at El Chaupi, Cantón Mejía, Ecuador were pooled and each point at disease onset plus 90, 105, and 120 days represents the mean of four independent repetitions.

D. Days after Sowing

These facts are consistent with our previous studies, where we found that induction of growth hormones and activation of enzymes associated with plant defense mechanisms, including peroxidase and superoxide dismutase in lupin was due to the application of native *B. subtilis* [15], [27] in addition to the production of lipopeptides that suppressed lupin anthracnose [16]. Previous evidence, together with those found in this study, suggest that native *B. subtilis* work in two of the three vertices of the disease triangle, first limiting the germination of spores and mycelium of *C. acutatum* and second inducing systemic resistance on lupin plant.

E. Estimation of bacterial population on the phylloplane of lupin

The size of *B. subtilis* population on the phylloplane of two anthracnose susceptible lupin cultivars estimated by the serial dilution - plating method is presented in Fig. 5a-5b. Viable cell concentration of *B. subtilis* was reduced CFU/g in 2.0 Log UFC/g after 15 days of the first application (Fig. 5A, B). A *B. subtilis* strain selection program generally begins with screening under controlled laboratory or semi-controlled greenhouse conditions; however, it is difficult to predict how the bacteria will respond when released into the natural environment [22]. In another study, *B subtilis* reduced very

little in strawberry leaves under greenhouse conditions, but its population size was reduced by 50% in the open field after 8 days of application [28]. The population of *B. subtilis* will vary naturally once it has been released in the field. Environmental stresses such as intense sunlight, dryness, and high temperature could reduce initial colonization [28], or rainfall could easily remove the bacterial population from the plant surface in several hours [29]. In this sense, it is important to maintain the stability of the antimicrobial substances produced by *B. subtilis* [16], but these metabolites are very complex and tend to be easily degraded by exposure to various environmental factors in the field [25].

Plants in addition release organic compounds as sugars, organic acids, and growth regulators [22] that can stabilize cell concentration of *B. subtilis* due to nutrient availability [28]. Thus, when bacterial antagonist adhered, invaded, and survived on leaves, they effectively prevent pathogen attaching and colonization [29]. Addition of 0.5% unrefined sugar to *B. subtilis* suspensions provide initial food supply and increase adherence and colonization of bacteria in cocoa pods improving monilia pod rot control [8] New strategies for application, colonization, and survival of native *B. subtilis* strains on the phylloplane are becoming increasingly important components to establish a sustainable integrated management of lupin anthracnose

Our results also indicate that the average of native *B. subtilis* population on the phyllosphere of lupin remains stable in around 7.0 Log UFC/g after every two-week spray and along the assessment for two months (Fig. 5a-5b) that indicate that after population became stable, *B. subtilis* resisted harsh environmental conditions. The serial dilution plating method is adequate for determining the total epiphytic population on the phylloplane considering that bacteria can colonize stomata, trichomes, vein endings, cell wall junctions or even they could be beneath the leaf cuticle [21]. Lupin stems and leaves sprayed with bacterial antagonists were consistently protected from natural infection of *C. acutatum* fungi. Control was evident in a significant reduction in AUDPC (Fig. 4a-4b) and suggest that native *B. subtilis* are more likely the cause of anthracnose reduction. Our results are in line with [30] where a single inoculation of *B. pumilus* CCIBP-C5 CF reduced banana sigatoka *Mycosphaerella fijiensis* by 33.6% and delayed progress of the disease by 21-28 days, compared with the control. Lower AUDPC values in comparison with the control indicates reduction of the initial inoculum or reduction of pathogen on the plant [30]. Disease reduction may be caused by the wide range of lipopeptides including fengycins, iturins, and surfactins that synthesizes *B. subtilis* and that act as antifungal antibiotic substances [16].

The biological control activity of native *B. subtilis* against lupin anthracnose may be associated with several direct and indirect mechanisms. In previous studies, applications of *B. subtilis* lipopeptides, together with endospores and vegetative cells produced in vitro, to roots of lupin seeds and seedlings [15] induced a significant increase in the expression of genes associated with defense mechanisms and plant growth [27]. By associating prior findings with the field observations of this study where infections and disease severity were reduced, we can infer that the biocontrol of native *B subtilis* was not only by damage of cellular structures (hyphae, mycelium, spores) of the pathogen, as occurred in the in vitro studies, but also by induction of systemic resistance (ISR) and promotion of plant growth mechanism (PGP). An innovative strategy for the integrated management of anthracnose in lupin could be the activation of ISR by *B. subtilis.* New studies will allow more efficient use of these native strains and perhaps allow the establishment of biocontrol strategies [8].

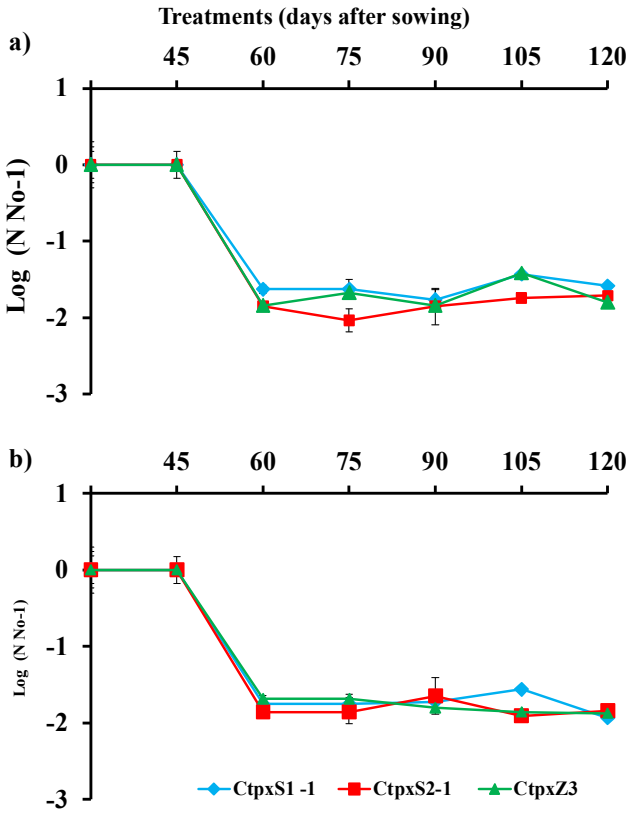

Fig. 5 Population of *B. subtilis* Ctpx-S1-1; Ctpx-S2-1 and Ctpx-Z3 recovered from the phyllosphere of lupin cultivars: a: I.450 Andino; b: I-451 Guaranguito during 2015 and 2019 growing seasons. First spray at disease onset and fortnightly for eight weeks. Sampling, culture and cell suspension as described in methodology. Colony counted and results expressed as CFU *B subtilis* per gram of lupin leaves. To improve homogeneity of variances, data of bacterial concentration were log 10-transformed (log CFU/g). Each point represents the mean ± SD of four independent repetitions of 10 g leaves.

Based on data of the present study and in previous works we suggest that bacterial suspensions $1x10^9$ UFC/ ml should be applied either as first symptoms appear or better prior pathogen infection. Success in controlling lupin anthracnose disease can be achieved with a sequential two-week application. Short interval applications of *B. subtilis* are often recommended, during the rapid growth stages of the plant, to obtain commercially acceptable efficacy [28]. Special attention should be considered on cotyledonal, flowering, and pod-filling stages because lupin as shown to be more anthracnose susceptible on these phenological stages [18].

Other technological components are needed to reduce the use of synthetic pesticides further. For this, it is necessary to develop *B. subtilis* at a semi-industrial or industrial level or to use the toxic substances extracted from bacteria within rotation plans with chemical products. These rotation plans would include the interaction of *B. subtilis*-based biopesticides with protective and systemic fungicides to determine dose, frequency, and times of application [31]. We

are conducting studies in the use of a low-cost media for *B. subtilis* lipopeptide production [32], [33] and spry drying formulation [32], [33] for the development of new application approaches and to expand survival and reproduction of the antagonist under environmental field conditions.

IV. Conclusion

The results of this experimental study demonstrated that native Andean *B. subtilis* that previously showed efficiency in controlling anthracnose on seed and seedlings exhibited the ability to act as efficient biocontrol agents against lupin anthracnose and thus can be used to suppress disease. *B subtilis* treatments highly decreased lesion diameter compared with the treatment inoculated with pathogen alone as time elapsed to 28 days after inoculation in two lupin anthracnose susceptible cultivars, in the greenhouse. Every two-week application of native *B. subtilis* strains at $1x10^9$ UFC/ml concentrations outweighed the deleterious impact of anthracnose on lupin plants in the open field. AUDPC of lupin anthracnose was significantly reduced due to applications of *B. subtilis* in the field trials. The viable cell population of antagonist bacteria was stable along the four assessments that suggest it is the cause of plant protection and disease reduction.

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