Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9275
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v36i4.18018

Is curtobacterium wilt biocontrol temperature dependent?

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ABSTRACT. Abiotic stress interferes with plant-microbial interactions, but some microorganisms may buffer this interference. We investigated the interaction between temperature and bacterial wilt (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens* - *Cff*) biocontrol and the ability of *Bacillus subtilis* strain ALB629 to colonize bean seedlings, to inhibit pathogen growth and to use different C and N sources. *B. subtilis* ALB629^{rif}, a mutant selected from the wild population of ALB629, was used to monitor plant colonization at 20°C and 30°C. ALB629^{rif} was detected only in the plant roots ($10^{3.22}$ CFU g^{-1}) at the lower temperature but colonized the roots, stems, and leaves ($10^{5.85}$, $10^{4.48}$, and $10^{4.01}$ CFU g^{-1}), respectively, at 30ºC. The area under the disease progress curve was also different at the two tested temperatures ($p < 0.01$). Nevertheless, the disease reduction using ALB629^{rif} - treated seeds was similar: 71% and 75%, respectively, at 20 and 30ºC ($p < 0.01$). A higher efficiency of C and N source utilization was observed at the higher temperature, but the antagonist inhibited *Cff* growth equally at either temperature *in vitro*. Based on our results, temperature interferes with pathogen and antagonist plant colonization, but the overall suppression of bacterial wilt appears to be stabilized by ALB629.

Keywords: seed treatment, *Phaseolus vulgaris*, antibiosis, temperature, rhizobacteria, PGPR.

O biocontrole da murcha-de-curtobacterium é dependente da temperatura?

RESUMO. Estresses abióticos interferem nas interações micro-organismo e, mas alguns micro-organismos podem amenizar estes efeitos. Foi investigada a interação entre temperatura e controle biológico da murcha-de-curtobacterium (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens* - *Cff*) e a capacidade de *Bacillus subtilis* isolado ALB629 em colonizar plântulas de feijão, inibir o crescimento do patógeno e usar diferentes fontes de nutrientes. *B. subtilis* ALB629^{rif}, isolado mutante selecionado da população selvagem de ALB629 foi utilizado para rastrear a colonização da planta a 20 e 30ºC. ALB629^{rif} foi detectado apenas nas raízes ($10^{3.22}$ UFC g^{-1}) na menor temperatura enquanto que a 30ºC, ALB629^{rif} colonizou as raízes, caules e folhas ($10^{5.85}$, $10^{4.48}$ e $10^{4.01}$ UFC g^{-1}), respectivamente. A AACPD foi diferente nas duas temperaturas testadas ($p < 0,01$). No entanto, a redução da doença obtida pelo tratamento de sementes com ALB629^{rif} foi semelhante de 71% e 75%, respectivamente a 20 e 30ºC ($p < 0,01$). Uma maior eficiência de utilização de fontes de C e N foi observada na temperatura mais elevada, mas o antagonista igualmente inibiu o crescimento de *Cff in vitro* em qualquer temperatura. Portanto, a temperatura interfere na colonização da planta pelo patógeno e pelo antagonista, mas a supressão da murcha-de-curtabacterium de modo geral parece ser estabilizado por ALB629.

Palavras-chave: tratamento de sementes, *Phaseolus vulgaris*, antibiose, temperatura, rizobactéria, BPCP.

Introduction

Common bean (*Phaseolus vulgaris* L.) is the most important legume in Brazil; the crop is cultivated throughout the year, mainly by small growers, with an estimated 10% utilizing certified seeds (SENA et al., 2008). Although various bean seed-transmitted pathogens exist, the bacterial pathogens are the most difficult to control, particularly if no resistant commercial cultivar is available. Presently, the most serious bean bacterial disease is bacterial wilt, which is caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) (Hedges) Collins and Jones (VALENTINI et al., 2010). The pathogen was reported for the first time in South Dakota, United States by Heges in 1922 (VENETTE et al., 1995) and rapidly spread worldwide. In Brazil, the disease was first reported in 1997 by Maringoni and Rosa (1997) and, thereafter, became a major problem of common beans in different regions. In spite of its relatively recent discovery in Brazil, the pathogen has already spread throughout most of the bean-producing fields (HERBES et al., 2008), causing high economically important losses.

However, disease control by employing resistant cultivars is not possible because there is no commercially available resistant cultivar to date, though there are some germplasm lines of common bean that have demonstrated different levels of resistance to the yellow and orange variants of *Cff* (HSIEH et al., 2005; THEODORO et al., 2007). Infected seeds represent an important source of inoculum for bean bacterial wilt (HSIEH et al., 2003), yet no chemical seed treatment is registered (MARTINS et al., 2013; HSIEH et al., 2004). Therefore, other methods, such as biological control, need to be employed as potential tools for the management of bacterial wilt in common bean, particularly if used as a treatment of contaminated seeds. Plant beneficial bacteria (PBB), such as *Bacillus subtilis* strains, are among the disease control strategies that could potentially be useful to control plant pathogenic bacteria (MEDEIROS et al., 2009). Once an antagonist colonizes the plant tissue, the biological control of pathogens can result from a combination of mechanisms, including the competition for nutrients and space, antibiosis, and induced systemic resistance, and can sometimes result in beneficial effects to the host plant (HARMAN et al., 2010; MEDEIROS et al., 2012). Considering the importance of seeds in the transmission of pathogens and the need to reduce fungicide loads in the environment, seed treatment may provide a practical and cost-effective strategy to reduce such seed-born pathogens as *Cff* (HUANG et al., 2007; MARTINS et al., 2013). Unfortunately, the efficacy of biological control may be strongly influenced by sudden changes in temperature, and its efficacy may be dependent on the stability of such environmental variables as temperature (ASHRAFUZZAMAN et al., 2009). Indeed, both microorganisms and plants are influenced by temperature changes, and the root exudates that support rhizobacterial growth are more rapidly or slowly released at a higher or lower temperature, respectively, thus impacting the growth of bacteria in the rhizosphere (KATO et al., 2005; NIHORIMBERE et al., 2009).

This work aimed to verify the potential of *B. subtilis* ALB629 seed treatment to prevent bacterial wilt at 20 and 30ºC and to study the relationship between these temperatures on the ability of ALB629 to colonize bean seedlings, to inhibit pathogen growth and to use different carbon and nitrogen sources, the major components of bean root exudates.

Material and methods

Selection of the B. *subtilis* ALB629^{rif} mutant

To study the antagonist colonization of bean seedlings, a naturally occurring mutant, ALB629^{rif}, was selected from a *B. subtilis* strain (ALB629) and comparatively tested for growth and biocontrol capacity with its wild-type counterpart. To generate a mutant that is resistant to rifampicin, increasing amendments of this antibiotic were added to the nutrient agar (NA) medium up to 100 ppm at each bacterial culture, similar to a previously described method (MEDEIROS et al., 2009). Both ALB629 and ALB629^{rif} were preserved in peptone glycerol at -80ºC until use.

Biocontrol of bacterial wilt by B. *subtilis* wild-type and mutant strains at two temperatures

In this first experiment, *B. subtilis* ALB629 and its mutant, *B. subtilis* ALB629^{rif}, were used as biocontrol agents against bacterial wilt at of 20 and 30ºC to ascertain the stability of the biological control activity of the mutant strain.

Artificial seed inoculation

Prior to inoculation, commercially available bean seeds (cv. Pérola) were disinfected by soaking in alcohol (70% ethanol) for 30 s, followed by sodium hypochlorite (0.5% active chloride) for 10 min. and sterile distilled water (SDW).

The *Cff* strain was isolated from dried infected leaves and cultivated on 523 Kado and Heskett medium in Petri dishes at room temperature (28ºC) for 48 hours. The bacterial cells were then transferred to 523 liquid medium in test tubes and cultivated for 48 hours at room temperature (28ºC) with shaking at 150 rpm. A 100 µL aliquot of the bacterial suspension was spread using a *Drigalski* spatula onto the surface of CNS medium amended with mannitol. The Petri dishes were placed at room temperature (28ºC) for 72 hours. The disinfected seeds were artificially inoculated with *Cff* by the physiological conditioning technique of Deuner et al. (2011). The seeds were air-dried in a laminar flow cabinet for 8 hours, and fifty seeds/Petri dish were arranged over the *Cff* growth for 48 hours at room temperature (28ºC), scraped from the medium and immediately treated as detailed below. Non-inoculated controls were incubated in the same medium under the same conditions without the pathogen.

Biological seed treatment to control bacterial wilt at two temperatures

B. subtilis ALB629 and its mutant ALB629^{rif} were cultivated on NA medium in Petri dishes at room temperature (28ºC) for 48 hours. Cells were

subsequently transferred to a nutrient stock medium and cultivated for 48 hours at room temperature (28°C) with shaking at 150 rpm. The endospore concentration of the bacterial suspension was adjusted using a Neubauer chamber to 1×10^8 CFU mL^{-1} by dilution plating and then used for the seed treatment. The inoculated seeds were individually treated with the *B. subtilis* wild strain (ALB629) and its mutant ($ALB629^{rif}$) in a suspension of 10^8 CFU mL^{-1}, copper oxychloride (2 g a.i. L^{-1}), or water by imersion at the ratio of 2 mL per gram of seed for 30 min. Seeds without *Cff* inoculation were immersed in water solution as a control. The suspension was rinsed off, and the seeds were sown in 3 L pots filled with Argissoil, with 6 seeds per pot. A portion of the pots were maintained in growth chambers at 20°C and another portion at 30°C under a 12 hours photoperiod. The experiment was arranged in randomized block, with four replicates, and the plants were watered to maintain the soil moisture at field capacity.

Assessment of the analyzed variables

The curtobacterium wilt severity was evaluted according to Hsieh et al. (2003) at 12, 15, 18, 21 and 24 days after sowing (DAS). A 0–5 scale was used, as follows: 0 = no wilt symptoms; 1 = wilt on one of the primary leaves; 2 = wilt on both primary leaves but not on the first trifoliolate leaf; 3 = wilt on the first trifoliolate leaf; 4 = death of the seedling after wilt development on the primary leaves and 5 = unmerged seedling or death of seedling before wilt development on the primary leaves. The data were used to calculate the area under the disease progress curve (AUDPC) according to Shaner and Finney (1977). The experiment was arranged in a randomized block design. The data were subjected to a variance analysis (ANOVA); for significant effects, the data were compared using Tukey's test ($p = 0.05$). The experiment was repeated twice.

Colonization of bean seedlings by $ALB629^{rif}$

In this second experiment, the artificial seed inoculation and the biological seed treatment with $ALB629^{rif}$ were performed, as described previously. To track plant colonization by the biocontrol agent, two plants per pot were randomly sampled at 24 DAS and separated into roots, stems and leaves. The cotyledons were used as the leaf samples, the stem samples consisted of the region between cotyledon insertion and the first true leaf, and the root samples consisted of the entire root system. Each assessed plant tissue was weighed, surface disinfected with alcohol (70%) for 30 s, followed by hypochlorite (0.5% active chloride) for 10 min. and washed twice with SDW. The tissue was then transferred to 10 mL tubes containing 5 mL of SDW; the tissue was crushed, vortexed for 1 min. and serially diluted to 10^{-5}. The crude extract and the 10^{-1} to 10^{-5} dilutions were then plated by spreading on NA medium with 100 ppm rifampicin and incubated at room temperature (28°C). Colonies were counted after 2 days of incubation, and the data were transformed to log10 CFU g^{-1} of fresh tissue. The experiment was performed in a randomized block design. The data were subjected to an ANOVA; for significant effects, the data were compared using Tukey's test ($p = 0.05$). The experiment was repeated twice.

Antibiosis *in vitro*

The stability of the *Cff* inhibition *in vitro* was tested at two temperatures: 20 and 30°C. The bacterial pathogen was cultivated in liquid medium 523 for 48 hours, and 100 μL of the suspension was spread on CNS medium. The antagonist was grown in liquid medium MCF, and a 10 μL aliquot of 10^8 CFU mL^{-1} of ALB629 and a 10 μL aliquot of MCF medium was transferred to plates containing the *Cff* suspension. Five plates were incubated at 20°C and another five at 30°C. The zone of inhibition was recorded 24 hours afterwards. The experiment was performed in a factorial scheme using a randomized block design. The data were subjected to an ANOVA, and the significant effects were compared using Tukey's test ($p = 0.05$). The experiment was repeated twice.

B. *subtilis* ALB629 growth using different C and N sources

In this experiment, *B. subtilis* ALB629 was tested for its ability to use different nutritional sources of carbon and nitrogen under at 20 and 30°C. Thus, ALB629 was grown in four different culture media for 12, 24, 36, and 48 hours to create a growth curve. The different culture media differed from each other by the following substrates: glucose, sodium citrate, aspartate, and glutamate, with the two first two being used as carbon sources and the last two used as nitrogen sources. These four different nutrients were used at 10 g L^{-1} concentration except for glutamate (15 g L^{-1}). Each of these nutrients was added to another solution composed of 0.5 g L^{-1} $MgSO_4$ ($7H_2O$), 1.0 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KCl, 1.0 g L^{-1} yeast extract, 1.2 mg L^{-1} $FeSO_4$ ($7H_2O$), 0.4 mg L^{-1} $MnSO_4$ (H_2O), 1.6 mg L^{-1} $CuSO_4$, and 1 g L^{-1} $(NH_4)_2SO_4$. The pH of each medium was adjusted to 7 with NaOH or 0.1 N HCl before sterilization, as described previously (NIHORIMBERE et al., 2009).

The experimental plot consisted of a test tube with 3 mL of each of the above 4 culture media, with three replicates per plot x time x temperature. To evaluate ALB629 growth, the strain was cultivated in a nutrient broth composed of 13.8 g L^{-1} yeast extract, 2.5 g $L^{-}K_2HPO_4$, 1.0 g L^{-1} KH_2PO_4 (anhydrous), 2.5 g L^{-1} NaCl, 6.5 g L^{-1} sucrose, 0.25 g L^{-1} magnesium sulfate and 0.1 g L^{-1} manganese sulfate for 48 hours at 28°C under stirring at 150 rpm. Every 12 hours (for 48 hours), a 150 μL aliquot of the antagonist suspension was transferred to each tube containing 3 mL of each previously described medium. The test tubes were placed in two different orbital shakers at different temperatures, 20 and 30°C, both at 150 rpm. After 48 hours from the first transfer, for each incubation temperature (20 and 30°C) and each sampled time point (12, 24, 36, and 48 hours after the onset of the experiment), three replicates of one tube each were individually analyzed by measuring the absorbance using a light spectrophotometer DU® 640B at λ = 600 nm. The experiment was performed in a factorial scheme (2 temperatures x 4 sampled time points x 4 nutrient sources) using a randomized block design. The data were subjected to a variance analysis (ANOVA); for significant effects, the temperature and nutrient factors were compared using Tukey's test (p = 0.05) and the sampled time points using a regression analysis. The linear regression equations were subjected to a parallelism (*F*-test) test to verify the null hypothesis that the slopes of the equations were statistically equal. The goodness of fit of the models was tested at 0.05 significance and evaluated by its coefficient of determination (R^2). The data analyses were performed using SISVAR software (FERREIRA, 2011).

Results and discussion

Disease control was achieved by seed treatment with *B. sutilis* ALB629 at approximately. 70% compared to the water control; both wild-type and mutant ALB629 displayed the same growth rate. Moreover, based on the area under the disease progress curve (AUDPC), the biological control of the bacterial wilt of bean was not different between the wild-type and mutant strains at 30°C (p = 0.8596) or 20°C (p = 0.1572), and both were effective in reducing the common bean bacterial wilt severity progress (AUDPC) at either temperature.

Because the wild-type and mutant strains showed the same disease reduction, all of the subsequent experiments were performed using only the mutant. Although the AUDPC was different between both tested temperatures (p < 0.01), the disease reduction obtained by the ALB629^{rif} seed treatment was similar at 71 and 75%, respectively, for 20 and 30°C when compared with the non-inoculated untreated seeds (control) (p < 0.01) (Table 1).

Table 1. Effect of seed treatment with the rifampicin-resistant strain of *Bacillus subtilis* ALB629 (ALB629^{rif}), copper oxychloride and water on the area under the disease progress curve (AUDPC)★ in the presence of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*).

Treatment	Temperature	
	20°C	30°C
ALB629^{rif}	64.95 bB★★	136.60 bA
Copper oxychloride	175.84 aB	497.40 aA
Cff-inoculated control	227.48 aB	554.35 aA
Non-inoculated, untreated control	0.00 bA	0.0 cA
[1]C.V (%) = 19.39		

★Calculated based on the disease score according to Hsieh et al. (2003) at 12, 15, 18, 21, and 24 days after sowing; ★★The means written with a common letter do not differ significantly according to Tukey's test (p ≤ 0.01); the lowercase refers to the column, and the uppercase to the row. [1]C.V. - Coefficient of variation. Means of two experiments of four replicates of six seedlings each.

Although *B. subtilis* ALB629^{rif} could efficiently reduce the disease severity at either temperature, it had a direct effect on the overall AUDPC (Table 1). At the higher temperature, a higher AUDPC was observed for all the treatments, and this corroborates previous findings for the influence of temperature on bacterial wilt severity (KRAUSE et al., 2009). Nevertheless, the biocontrol agent ALB629 induced a consistent disease reduction of approximately. 72%, regardless of the temperature. The same temperature-independent trend was not observed for the seeds treated with copper oxychloride (p < 0.001), the putative positive control.

The mutant strain, which showed consistent disease control, was used to monitor plant colonization. The plants were colonized by the bacterium, regardless of the temperature; however, the bacterium colonized the roots ($10^{5.85}$ CFU g^{-1}), stems ($10^{4.48}$ CFU g^{-1}) and leaves ($10^{4.01}$ CFU g^{-1}) at 30°C, whereas it remained confined to the roots ($10^{3.22}$ CFU g^{-1}) at 20°C, with a population approx. 100 times smaller. Because the pathogen was not as damaging at the lower temperature, the antagonist was able to control the disease, even at a less robust plant colonization (Figure 1).

ALB629 was confined to the roots when the plants were grown at 20°C and colonized the entire plant at 30°C, suggesting that the induced resistance might play a pivotal role as a control mechanism in this plant-pathogen interaction (ONGENA et al., 2007). Future experiments will investigate the activity of key defense-related enzymes in disease control; although it might also be present at the higher temperature, direct antibiosis will still have to

be considered as a key mechanism to cope with more aggressive plant colonization under this condition. Another hypothesis that may be drawn to explain the observed disease control at the lower temperature is that because both the pathogen and biocontrol agent were introduced via seeds, both shared the same niche at the early stages of plant development. Thus, an initial direct antibiosis may have occurred, reducing the initial inoculum of the pathogen, and, even when ALB629 was confined to the roots, the possible induced resistance triggered by the biocontrol agent or simply by the low pathogen inoculum would be sufficient to cope with the disease. We are presently investigating each of hypotheses to explain the observed disease control.

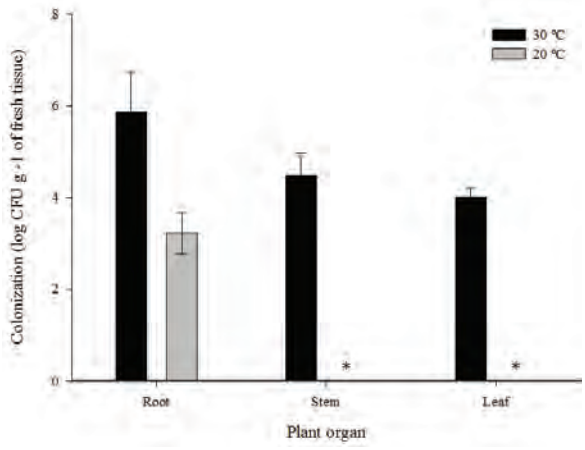

Figure 1. Colonization of bean seedlings by *Bacillus subtilis* ALB629^{rif} inoculated by seed immersion at a concentration of 10^8 CFU mL^{-1} for 30 min. *n.d.: not detected by the plate dilution method. The vertical bars represent the standard error of the mean. Means of two experiments of four replicates of six seedlings each.

The diversity of the root exudate secreted at each temperature may also regulate the rate at which ALB629 was able to grow. Bean roots release large amounts of amino acids and sugars (ODUNFA, 1979), which supply the demands of carbon and nitrogen for rhizosphere microorganisms (YOUSSEF; MANKARIOS, 1968).

Based on the spectrophotometric absorbance reading (λ = 600 nm), a different pattern of ALB629 growth was observed when it was cultivated using different nutrient sources ($p < 0.01$). Moreover, the bacterial growth at 30°C was higher than at 20°C ($p < 0.01$) (Table 2).

The nutrient sources supported different bacterial growth at each temperature. Glutamate supported the highest growth, which was more than two times higher than the growth observed for aspartate, the nutrient source that supported the second-highest growth. In contrast, sodium citrate supported a comparatively lower growth, but the growth was higher than with glucose. For all of the nutrient sources except aspartate, the growth observed at 30°C was higher than that observed at 20°C.

Table 2. *Bacillus subtilis* ALB629 growth in media with different C and N nutrient sources at temperatures of 20°C and 30°C.

Treatment	Temperature	
	20°C	30°C
Glucose	0.15 d B*	0.45 d A
Sodium citrate	0.40 c B	0.55 c A
Glutamate	1.25 a B	1.36 a A
Aspartate	0.59 b A	0.58 b A
[1]C.V (%) = 2.09		

*The means of absorbance followed by the same uppercase letter in a row and lowercase letter in a column are statistically similar according to Tukey's test ($p \leq 0.01$). [1]C.V. - Coefficient of variation.

Moreover, the bacterial growth differed over time ($p < 0.01$), with the growth increasing (12, 24, 36, and 48 hours) when the times were compared using Tukey's test (Figure 2A, B, C and D).

Except for sodium citrate and aspartate at 30°C, all of the other substrates differed significantly according to the *F*-test ($p < 0.05$) including sodium citrate and aspartate at 20°C. All the tested amino acids are components of bean root exudates and, compared to other sugars and amino acids, support higher surfactin, a lipopeptide that acts in the induction of resistance, production by *Bacillus subtilis* (NIHORIMBERE et al., 2009). It remains unknown whether the molecules produced by ALB629 directly or indirectly inhibit the growth of the pathogen. However, with regard to disease control independently of the temperature, the growth using aspartate was similar at either tested temperature, and the bacterial growth displayed a standard characteristic of a probable phase adaptation of twelve hours, a logarithmic phase at 12-24 hours, a stationary phase at 24-36 hours, and a decline phase thereafter. These results suggest that resistance induction plays an important role in the protection of bean against bacterial wilt triggered by ALB629. Future studies will determine the induction of resistance at different temperatures and lipopeptide production under these conditions. Furthermore, induced resistance elicitors may be responsible for the observed biological control because antibiosis at either temperature displayed similar results ($p < 0.05$).

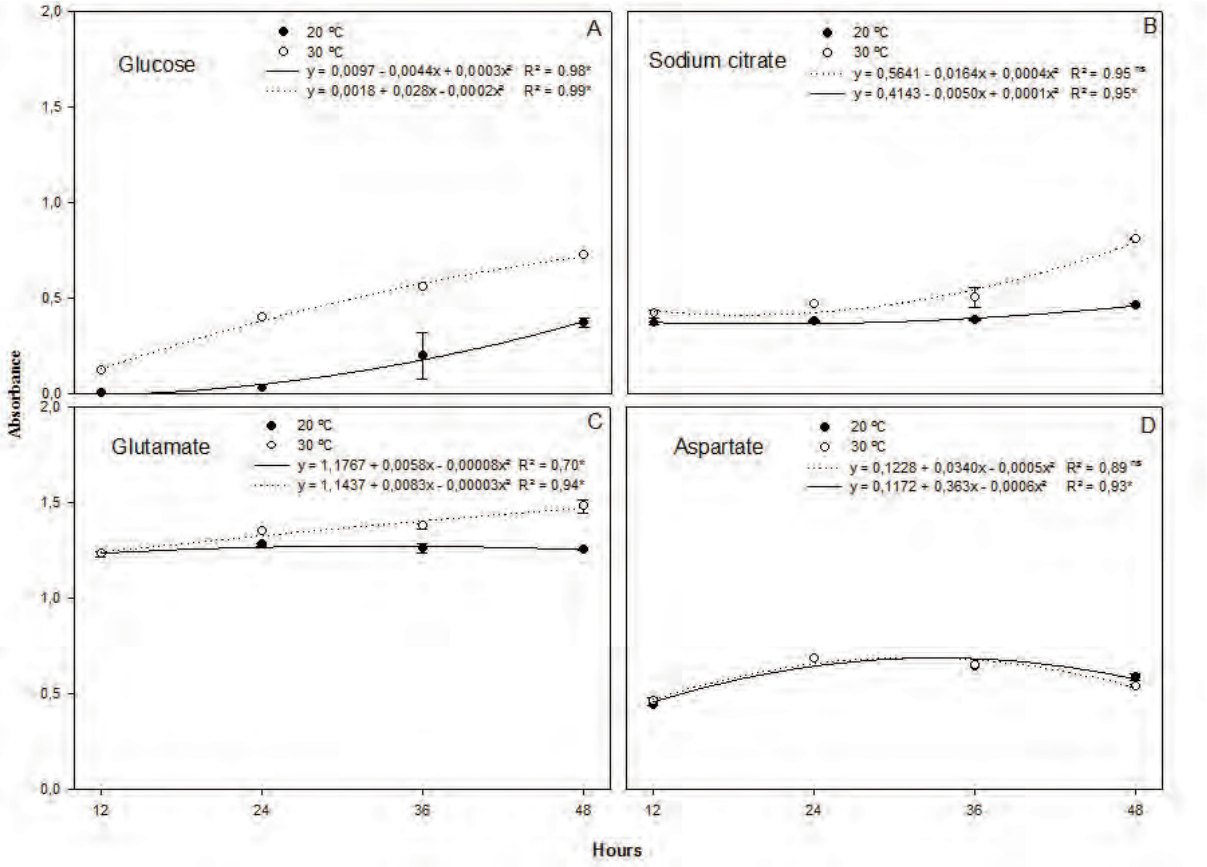

Figure 2. *F*-test comparing the linear regression angular coefficients derived from the *Bacillus subtilis* ALB629 growth in media with different nutrient sources and at temperatures of 20 and 30°C. The graphs in 2A, B, C, and D represent media with glucose, sodium citrate, glutamate, and aspartate, respectively. The vertical bars represent the standard error of the mean. ^{ns}: angular coefficients for ALB629 growth do not differ significantly. *Angular coefficients for ALB629 growth differ significantly ($F_{calculated} > F_{tabulated}$).

Therefore, *B. subtilis* ALB629 controls bacterial wilt in bean via treatment of infected seeds, and the stability of the disease control at different temperatures reinforces the potential practical use of this biological control agent, particularly because bean is cultivated throughout the year at different locations where different temperature conditions are found.

Conclusion

The stability of bacterium wilt control, the root colonization, the use of different nutrient sources and antibiosis at 20 and 30°C implies ALB629 as a promising tool to be used in the disease management in the field avoiding important bacterial wilt outbreaks that have been frequently observed in Brazil.

Acknowledgements

We thank Conselho Nacional de Desenvolvimento Cientifico Cultural (CNPq), Fundação de Apoio à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Programa de Apoio a Primeiros Projetos (PAPP/UFLA) for providing the financial support necessary for the development of this work. We thank CNPq for providing assistantship for the second and third authors.

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Received on July 20, 2012.
Accepted on October 24, 2012.