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ORIGINAL RESEARCH ARTICLE

Blackberry (*Rubus glaucus* Benth.) fertilized with nitrogen, phosphorus, potassium and calcium: Effect on anthracnose under controlled conditions

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ABSTRACT

In order to seek management alternatives for anthracnose caused by the fungus Colletotrichum gloeosporioides in blackberry (*Rubus glaucus* Benth.), at the Tibaitatá Research Center of the Colombian Agricultural Research Corporation AGROSAVIA (formerly CORPOICA), an experiment was conducted to evaluate the effect of the application of the major elements nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) on infections of the fungus *C. gloeosporioides* strain-52. For this purpose, a randomized complete block design was used with an arrangement of treatments in an orthogonal central composite design. To evaluate the relationship of fertilization levels and disease severity, an artificial inoculation was made on thorny blackberry stems using 0.5 cm mycelial discs at a concentration of 9.53 × 104 conidia. Observations consisted of: disease severity (S), incubation period (IP) and rate of development (r). Data analysis was done by the cluster method on the severity variable, a Pearson correlation analysis between variables, as well as a regression to estimate the effect of nutrients applied on the severity of *C. gloeosporioides* strain-52. The treatments were concentrated in four groups with the ranges (in parentheses) S (15.9% and 91.8%), PI (9 and 15.3) and Tr (0.0254 and 0.0468). A positive and significant correlation was observed between S and r ($P < 0.001$) and a negative correlation between PI with S and r ($P < 0.001$). By means of regression analysis, a linear model was generated that showed a reduction in disease severity with increasing N dose and an increase with the levels of P and Ca applied.

Keywords: *Colletotrichum Gloeosporioides*; Blackberry; Nutrition; Severity; Development Rate; Major Elements

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1. Introduction

In 2017, blackberry (*Rubus glaucas* Benth.) crops in Colombia occupied about 14,589 ha, with a production, on average, of 123,175 t[1]. In the phytosanitary field, crop production is threatened by various diseases, among which Anthracnose caused by the fungus Colletotrichum gloeosporioides stands out as the most limiting, since it generates losses close to 100% of the crop when preventive measures are not applied[2].

In Colombia, *C. gloeosporioides* and *C. acutatum* have been considered as causal agents of anthracnose on blackberry, and there are also reports on the attack of C. boninense[2–4]. Colletotrichum species show preferences for plant tissues; thus, *C. gloeosporioides* is associated to a greater extent with stem lesions, showing high growth rates and pathogenicity[3]. The management of *Colletotrichum* species has been on the application of fungicides. This type of management presents problems due to the development of resistant strains[5]. The abuse of chemical molecules for the control of Anthracnose in blackberry cultivation has caused the rejection of pulps for export, due to traces found in them[6].

Based on the above considerations, control methods have been studied for disease management through the application of biological products such as plant extracts[6], evaluation of promising materials[7] and characterization of resistant germplasm[3].

In this context, fertilization is beneficial to the reduction of disease incidence and severity[8], due to plant resistance and/or predisposition factors, generated by a nutritional imbalance[9]. In this sense, nutritional deficiencies can increase susceptibility to various diseases[10], while weak infections can become more aggressive[11].

Nitrogen (N) has been the most studied element in relation to host nutrition and disease prevalence, considering its essentiality in plant growth, its limited availability in the soil and its effect on cell size and cell wall thickness[12,13]. Additionally, it plays a fundamental role in plant-pathogen interaction, due to the mobility of the element during biotic and abiotic stress as a plant defense strategy[14].

Phosphorus (P) increases plant resistance to different diseases when its concentration in tissues accelerates maturity and protects young organs; however, the effects on disease severity are not as evident when observed in susceptible and/or moderately resistant cultivars[15].

Potassium (K) is the most abundant inorganic solute in plant cells and is related to plant response to abiotic and biotic stresses[16] and its deficiency causes reduction in the synthesis of proteins, starch and cellulose (high molecular weight compounds) and therefore induces the accumulation of low molecular weight organic compounds that can cover nutrients required by microorganisms[8].

Various fungi invade plant tissues by producing pectolytic enzymes, such as polygalacturonase, which dissolve the middle lamella of cells; however, the activity of this enzyme can be inhibited when the concentration of calcium (Ca) in the tissues increases, while contributing to the formation of protective physical barriers against the penetration of these organisms[17].

In accordance with the above considerations, the objective of the present research was to evaluate under greenhouse conditions the effect of N, P, K and Ca fertilization on the development of *C. gloeosporioides* strain-52 in blackberry plants with thorns.

2. Materials and methods

2.1 Location

The experiment was conducted in the greenhouse of the Tibaitatá Research Center of the Colombian Agricultural Research Corporation AGROSAVIA (formerly CORPOICA), located at kilometer 14, Bogotá-Mosquera Road, at 2,600 m.a.s.l., in the department of Cundinamarca, at 4°42′ N and 74°12′ W, with an average temperature of 13.1 °C, relative humidity of 80%, at 2,600 m.a.s.l.

2.2 Application of treatments

Table 1. Composition of treatments applied to blackberry Castilla seedlings inoculated with *C. gloeosporiodes* strain-52

Treatment	**Nutrient applied (g/plant)**			
(no.)	**N**	**P_2O_5**	**K_2O**	**CaO**
1	16	12	24	12
2	48	12	24	12
3	16	57	24	12
4	48	57	24	12
5	16	12	72	12
6	48	12	72	12
7	16	57	72	12
8	48	57	72	12
9	16	12	24	28
10	48	12	24	28
11	16	57	24	28
12	48	57	24	28
13	16	12	72	28
14	48	12	72	28
15	16	57	72	28
16	48	57	72	28
17	9	35	48	20
18	55	35	48	20
19	32	3	48	20
20	32	66	48	20
21	32	35	14	20
22	32	35	82	20
23	32	35	48	9
24	32	35	48	31
25	32	35	48	20

Based on fertilization studies carried out by Vayas and Artunduaga, 25 treatments with different doses of the nutrients N, P, K and Ca (**Table 1**) were defined and applied to 10-month-old blackberry seedlings with spines and phenotypic uniformity, which were sexually propagated and planted in plastic bags with 1,500 g of substrate, composed of a mixture of peat and rice husks, in a 3:1 ratio. The total amount of the elements per treatment was dosed weekly, preparing nutrient solutions from nitric acid, calcium chloride dihydrate, potassium chloride, ammonium diphosphate, magnesium sulfate heptahydrate, calcium nitrate tetrahydrate, and ammonium diphosphate, calcium nitrate tetrahydrate, potassium nitrate, potassium sulfate and sodium hydroxide, preserving the original concentrations of Mg, S and microelements of the nutrient solution of Hoagland and Arnon.

2.3 Pathogen inoculation

Strain-52 of *C. gloeosporioides* isolated from blackberry stems from Castilla, which was morphologically and molecularly characterized by AGROSAVIA in 2012[18], was used in the inoculation. This strain corresponds to an isolate obtained from blackberry stems of the San Antonio genotype, collected in the department of Antioquia (Colombia) and on which severities by C. *gloeosporioides* are reported between 10% and 100%, after 19 to 55 days from sowing. The reactivation was carried out in PDA culture medium in the Agricultural Microbiology laboratories of AGROSAVIA, Tibaitatá Research Center. Fifty-three days after the beginning of the experiment (DDCE), an artificial inoculation of the fungus was carried out on the stems, with discs of mycelium at a concentration of 9.53 × 104 conidia.

2.4 Evaluation of the disease

For this evaluation, a diagrammatic scale of severity in blackberry stems was elaborated (**Figure 1**) using as reference the scale proposed by López-Vásquez *et al.*[7], by means of which disease severity (S) was estimated with the following equation (equation 1):

$$S\ (\%) = \frac{\Sigma(n*b)}{N} \qquad (1)$$

Where: S (%) = severity of the disease expressed in percentage of affected tissue; n = number of sampling units classified in each grade; b = degree of severity; N = total number of sampling units observed.

Once S was identified, the rate of disease development (r) was calculated using the following equation[19]:

$$r = \frac{1}{t_1 - t_0}\left(Log_e \frac{X_1}{1-X_1} - Log_e \frac{X_0}{1-X_0}\right) \qquad (2)$$

Where: r = rate of development; t_1 = final time; t_0 = initial time; X_1 = final severity; X_0 = initial severity.

With the data obtained, the incubation period (IP) was calculated, defined as the number of days elapsed from inoculation until the stems of the plants showed symptoms of anthracnose, which were manifested by the presence of purple to dark brown spots and dark edges on the inoculated tissue.

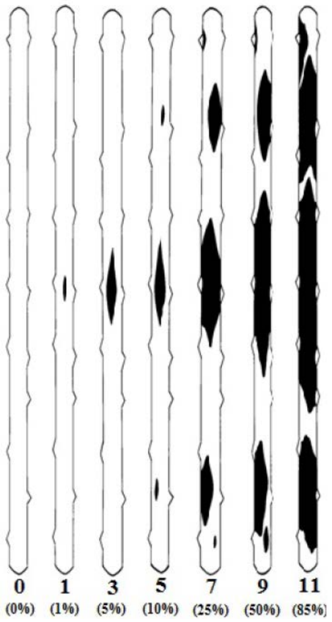

Figure 1. Proposed and used anthracnose severity scale for blackberry stems inoculated with agar discs (modified from López-Vásquez *et al.*[7]). 0: No apparent lesion or 0% of the tissue affected; 1: 1% of the affected tissue; 3: 5% of the affected tissue; 5: 10% of the affected tissue; 7: 25% of the tissue; 9: 50% of the affected tissue; 11: 85% of the affected tissue.

2.5 Experimental design and statistical analysis

A randomized complete block experimental design was used with an orthogonal central composite design treatment arrangement and three replications, for a total of 375 seedlings (25 × 5 × 3). A cluster analysis was performed for the severity variable, using the Cluster procedure of the Statistical Analysis System (SAS® 9.3) software, in order to classify the treatments into homogeneous groups. The level of association between the variables severity (S), development rate (r) and incubation period (IP) was analyzed using Pearson's correlation coefficient, verifying positive or negative relationships and degree of significance ($P < 0.001$). To estimate the effect of fertilization levels on disease severity, a regression analysis was used and the respective graphs were constructed using the free software R (R Foundation for Statistical Computing).

3. Results and discussion

3.1 Effect of treatments

Cluster analysis made it possible to classify the treatments into four groups according to the proposed statistics (**Figure 2** and **Table 2**).

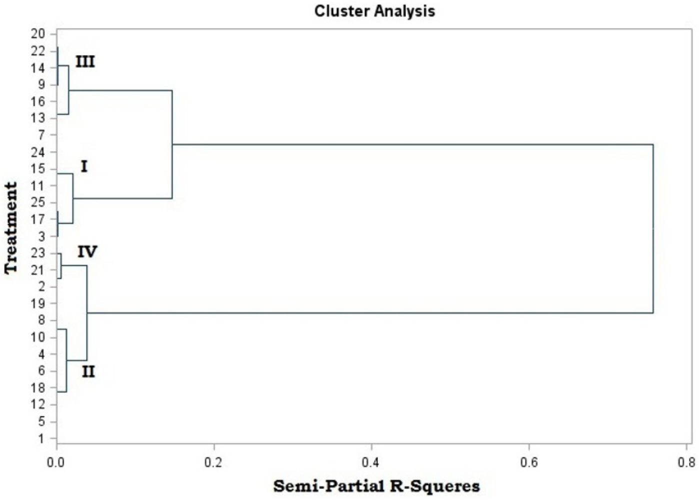

Figure 2. Grouping of treatments applied to blackberry seedlings based on the evaluation of severity, incubation period and development rate of *C. gloeporioides* strain-52.

Table 2. Severity groupings (S), incubation period (IP) and disease development rate (r) of *C. gloeosporioides* strain-52 inoculated on blackberry stems

Groups	Treatment (No.)	Dosage (g/plant)				Variable	Media	C.V.
		N	P_2O_5	K_2 O	CaO			
1	3	16	57	24	12	S	91.8	9.6
	11	16	57	24	28			
	15	16	57	72	28	r	0.0468	0.0
	17	9	35	48	20			
	24	32	35	48	31	PI	9.0	18.5
	25	32	35	48	20			
2	1	16	12	24	12			
	4	48	57	24	12	S	33.5	16.5
	5	16	12	72	12			
	6	48	12	72	12			
	8	48	57	72	12	r	0.0281	7.4
	10	48	12	24	28			
	12	48	57	24	28			
	18	55	35	48	20	PI	14.4	31.5
	19	32	3	48	20			
3	7	16	57	72	12	S	62.9	11.3
	9	16	12	24	28			
	13	16	12	72	28			
	14	48	12	72	28	r	0.0368	10.0
	16	48	57	72	28			
	20	32	66	48	20	PI	14.1	29.2
	22	32	35	82	20			
4	2	48	12	24	12	S	15.9	47.1
	21	32	35	14	20	r	0.0254	6.6

23	32	35	48	9	PI	15.3	35.9

Accordingly, the groups had an S between 15.9% and 91.8%, an r between 0.0254 and 0.0468 and a PI between 9 and 15.3 days (**Table 2**). In group 1 (G1), six treatments had the highest mean values of S and r, as well as the lowest mean value for PI. The best response on the evaluated variables was observed in group 4 (G4), composed of three treatments; while in groups 2 (G2) and 3 (G3) there were nine and seven treatments, respectively; with S values between 33.5 and 62.9, PI between 14.1 and 14.4 and r between 0.0281 and 0.0368 (**Table 2** and **Figure 2**).

3.2 Development of the disease

During the greenhouse work, the average temperature was 19 °C and relative humidity was 71%. Prior inoculation with strain-52 of *C. gloeosporioides*, 53 DDCE, the average temperature was 19 °C and decreased to 18 °C after 62 DDCE, while the relative humidity in both periods was, on average, 57%. Research in field conditions showed that temperatures of approximately 16 °C and relative humidity above 85% are appropriate for the increase of Anthracnose lesions on blackberry[20], which differs from the data recorded in the present work.

However, field studies with blackberry materials exposed to natural inoculations with *C. gloeosporioides*[7] show no interactions between the development of the disease and the percentage of environmental humidity, which is in accordance with the results observed in the present investigation, since the values reported in the epidemiology of the pathogen are not registered. In the aforementioned investigations, a relative humidity of 76.34% was recorded, with minimum and maximum values of 63.6% and 87%, respectively, conditions in which the genotypes susceptible to anthracnose showed severity between 45.6% and 50% and incidence between 79.8% and 99.4%.

Pearson's correlation analysis showed a positive and significant correlation between S and r ($P < 0.001$), and a negative and significant correlation between PI with S and r ($P < 0.001$) (**Figure 3**), indicating that increasing disease severity increases the rate of development, while increasing the incubation period decreases the severity and rate of development.

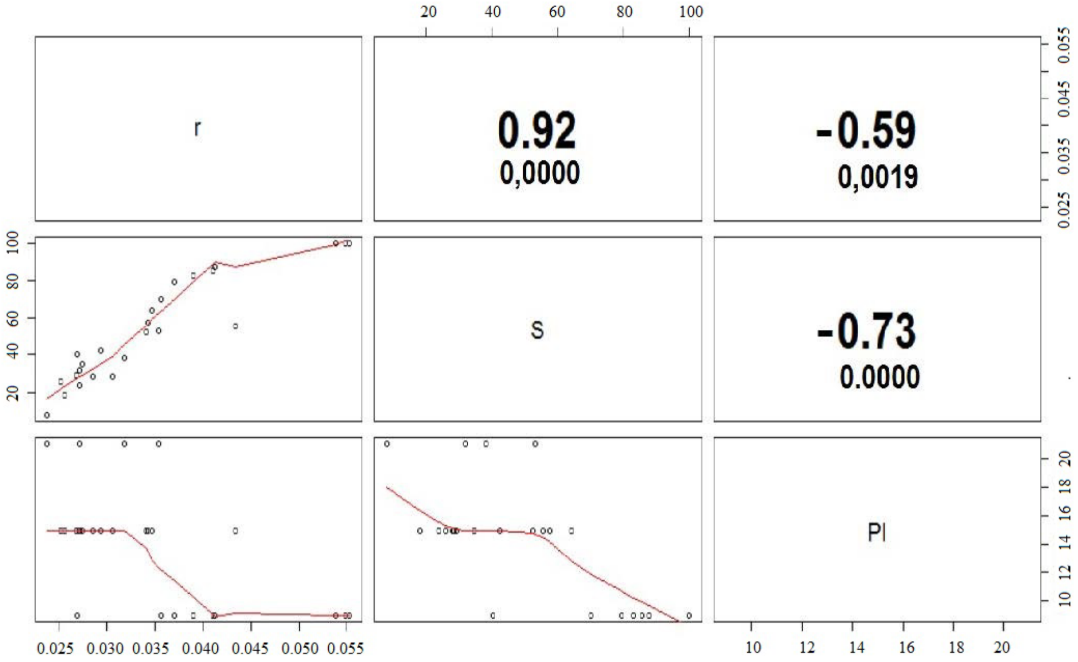

Figure 3. Relationships between development rate (r), severity (S) and incubation period (IP) in blackberry plants inoculated with *C. gloeporioides* strain-52.

The curves of disease development in the identified groups (**Figure 4**), allowed observing in G1 an exponential growth until 40 days after inoculation (DAI), followed by a linear growth until the end of the experiment (64 DAI) in all blackberry plants that received the fertilization treatments (T), except in those plants of T15 and T24. These treatments presented an exponential growth until 32 DAI, which became linear at 48 DAI and stabilized when reaching 100% severity at 110 DAI (**Figure 4**).

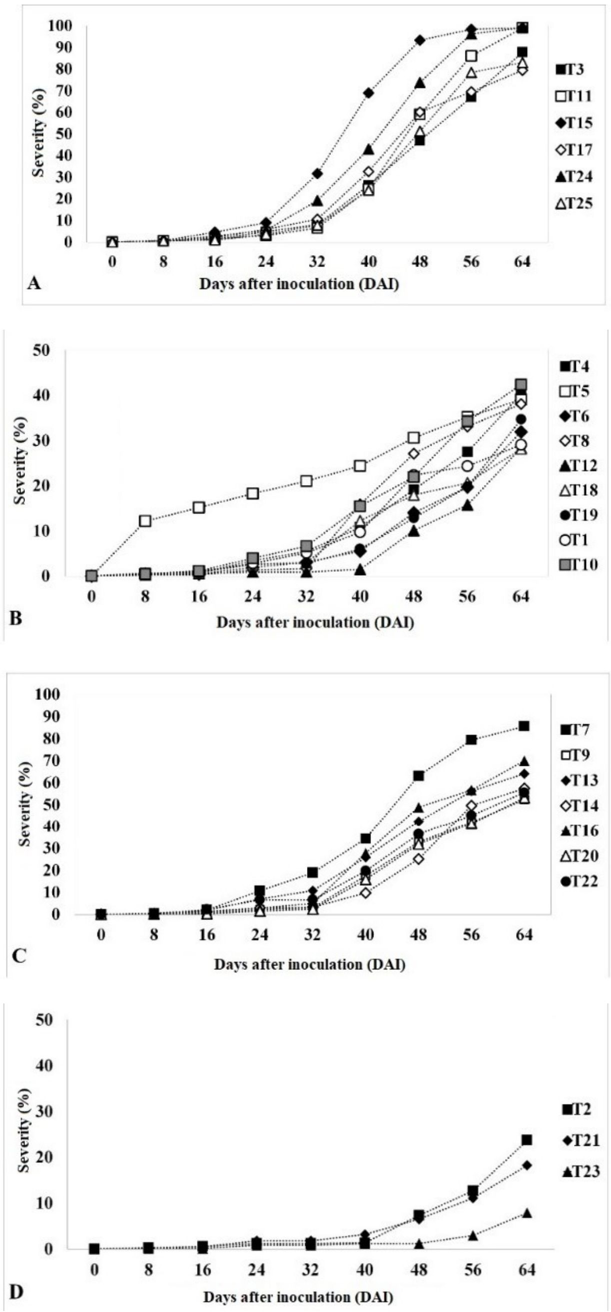

Figure 4. Development curves of *C. gloeosporioides* strain-52 on blackberry stems in groups 1 (**A**), 2 (**B**), 3 (**C**) and 4 (**D**).

In plants included in G2 and G3, exponential growth was observed up to 40 DAI, becoming linear up to 64 DAI, except for T5 and T7 in G2 and G3, respectively. In this sense, disease development in T5 presented a logistic growth from 8 DAI until the end of the evaluations, while T7 presented an exponential growth up to 32 DAI, which became linear and stable between 56 and 64 DAI (**Figure 4**).

In G4, disease development in plants that received fertilization treatments T2 and T21 showed exponential growth from 16 DAI until the end of the evaluations, while the disease in T23 plants showed logistic growth from 40 DAI, which did not exceed 10% severity 64 DAI (**Figure 4**).

According to the nutrients applied, the G1 treatments received doses of all elements, with the exception of N, K and Ca in T17, T15, T3 and T24, respectively. On the other hand, in the group that presented the best response (G4) there was greater variability in the doses applied, since there was only coincidence for N and P in T21 and T23, with the lowest dose of Ca in T23 (**Table 2**).

3.3 Effect of nutrients

Under the conditions of the present study, the regression analysis allowed fitting a multiple linear model (equation 3), where N has a negative effect on disease severity, i.e., with an increase of 1 g of this element, a decrease of 0.881% in disease severity is expected. For P and Ca, the opposite (positive) effect was observed, i.e., by increasing the content of these nutrients by 1 g, increases of 0.054% and 1.773% in disease severity occur, respectively (**Figure 5**).

$$\boldsymbol{Severity} = 27.371 - 0.881\mathrm{N} + 0.054\mathrm{P} + 1.773Ca \; R^2 = 0.5922 \tag{3}$$

The results with N application differ from the findings of Nam *et al.*[21], who found that high concentrations of this element increase the severity of *C. gloeosporioides* on strawberry plants (*Strawberry* cv. Nyoho); which in turn demonstrates a marked difference in the interaction processes between pathogens and hosts under conditions of nutritional imbalance. In this context, some foliar pathogens penetrate, multiply and develop in succulent tissues thanks to nitrogen supply[22].

However, these procedures expose plants to nutritional deficiencies and imbalances due to physiological patterns of plant-pathogen interactions, since generally the form of N available to the host or microorganism has a greater influence than the amount of the element applied[12].

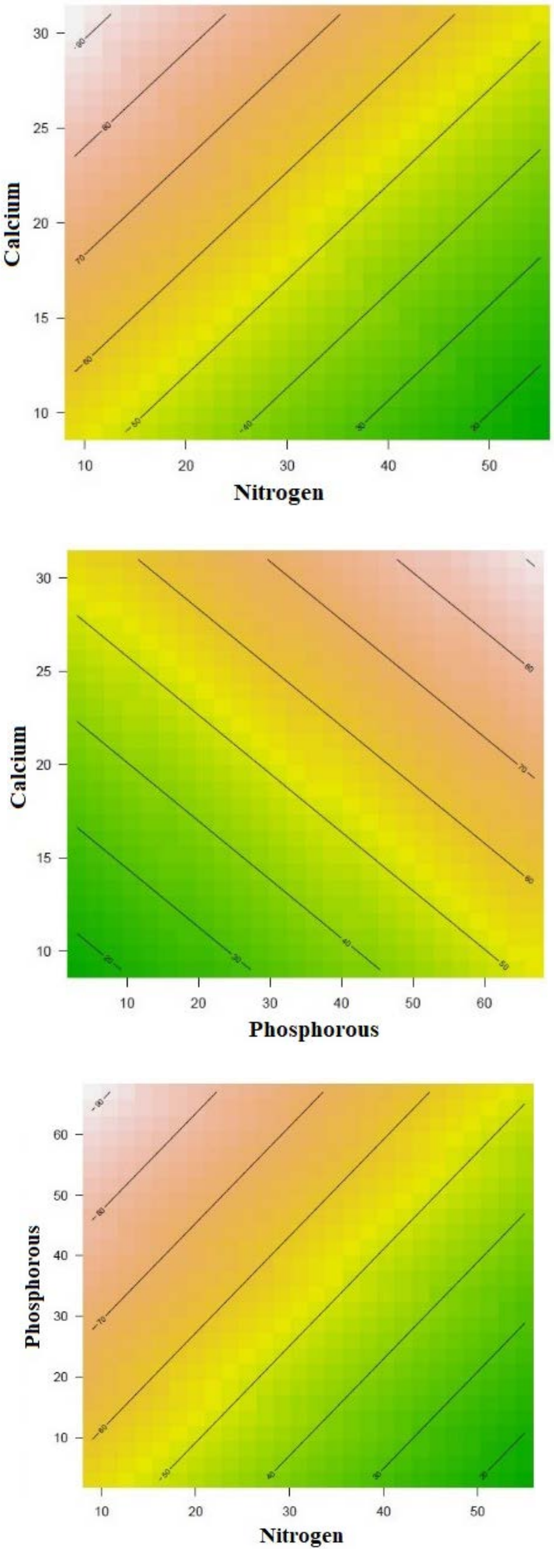

Figure 5. Contour plot for the multiple linear model of the effect of N, P and Ca on the percent severity of *C. gloeoporioides* strain-52 on blackberry stems.

The effect of P in the reduction of diseases is attributed to physiological processes that lead young tissues to infections by the maturation of organs; however, the effect is not noticeable when observed in susceptible and/or moderately resistant cultivars[15]; which coincides with the results obtained in the present research, considering the high pathogenicity of the strain used[18], the susceptibility of the material used, as well as reports on the influence of the element in the increase of diseases caused by various pathogens[15].

Despite the positive effect of Ca in disease management and its mechanism of action on pathogens[23], in the present study it could be inferred that the increase of this element contributed to the increased severity of *C. gloeosporioides* strain-52. In this sense, molecular studies show negative effects of Ca in the early stages of *C. gloeosporioides* infection, such as conidia germination and appressoria formation.

However, studies by Ahn *et al.*[24] demonstrated that exogenous addition of $CaCl_2$ restores conidial germination and appressoria formation, largely due to the expression of the Calmudulin gene (CgCaM), which was encoded by Polymerase Chain Reaction (PCR) methods and genomic DNA identification, as a regulator of biochemical and structural processes in *C. gloeosporioides.*

Although no significant effect of K on disease severity was evidenced in the present study, it is known that imbalances of this element influence physiological and biochemical processes that are relevant to plant susceptibility to pathogens. In this context, this nutrient induces microscopic defense responses in tissues, such as the accumulation of phenolic-type compounds and the formation of antifungal barriers[25]. However, the excessive increase in potassium concentration has no effect on the synthesis of high molecular weight compounds nor an additional effect on resistance and/or tolerance; since the addition of the element is only efficient in disease control only if its deficiency is covered[8].

4. Conclusions

Doses of 32, 35, 48 and 9 g/plant of N, P, K and Ca, respectively, applied to blackberry Castilla plants, caused a reduction in the severity and rate of development of *C. gloeosporioides* strain-52 and increased the incubation period of the fungus. A reduction in disease severity was observed with increasing N doses and an increase in disease severity with increasing P and Ca levels.

The results of the study showed the interaction between blackberry plants and strain-52 of *C. gloeosporioides*, the causal agent of anthracnose under conditions of nutritional imbalances, results that were possibly affected by the susceptibility of the plant material used and the high pathogenicity of strain-52.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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