Resistence induction in Brassica oleracea var. acephala to xanthomonas campestris pv. campestris and growth promotion by endophytic bactéria

Indução de resistência em Brassica oleracea var. acephala à Xantomonas campestris pv. campestris e promoção de crescimento por bactérias endofíticas

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ABSTRACT

Among the most economically expressive agricultural crops, cabbage (Brassica oleracea var. acephala) stands out as one of the most popular in human feed. However, Brazilian production has been suffering from the incidence of pests and diseases that affect the crops, and black rot of crucifer (BRC), the main cause of economic losses of its producers. In view of the above, the objective of this study was to identify endophytic bacteria to induce resistance to Xanthomonas campestris pv. campestris, causal agent of BRC, and promoting plant growth in B. oleracea. The experiments were conducted at the Laboratory of Microbiology of the Agricultural Sciences Center of the Federal University of Alagoas in Rio Largo/AL, Brazil. B. oleracea plants was cultivated with inoculation of five bacterial endophytes (ISO31, ISO33, ISO34, ISO48 and ISO51) in vases containing natural and sterilized soil for lesion estimation of in vivo pathogen and biometrics agronomic characteristics of production. The pathogen was inoculated with aid of a disposable syringe in the leaves. The data was subject to analysis of variance and the Scott Knott Test (p≤0,05). Isolate 34 showed the best performance in the promotion of resistance and plant growth in comparison to the other isolates, not showing significant interferences of the BRC in the development of the inoculated plants and in the biocontrol of the disease in the field.

Keywords: black rot, crucifer, biological control, beneficial bacteria.

1. INTRODUCTION

With its center of origin on the Mediterranean Coast (BALKAYA; YANMAZ, 2005), kale, cabbage or common cabbage (Brassica oleracea var. acephala) is a typical vegetable of mild or cold climate (between 16-22 ºC), annual or biennial cycle (NOVO et al., 2010), herbaceous and erect, being able to reach approximately 40 to 120 cm in height, presenting simple leaves, light green, long petiole, limbus and well developed veins and phylloxia rosulate around its stem (FILGUEIRA, 2003).
For its commercialization, several physical and sensorial attributes are evaluated by the producers and their respective consumers, aspects that help to ensure the receptivity of the food by the market, such as appearance, size, shape, brightness and leaf color (NOVO et al., 2010). However, some diseases directly interfere with the quality of the product, generating economic losses for the farmer and consequently reducing the quantity offered in the markets, supermarkets and free markets.

Among the most common bacterioses in cruciferous plants, black rot of crucifer (BRC), disseminated by the phytopathogen Xanthomonas campestris pv. campestris (Pammel) Dowson (Xcc), and the development of cuneiform lesions on the leaf edges, and in particular, the development of lesions on the foliar borders. the total senescence of the plant as a result of infection (MARINGONI, 1997; BEDENDO, 2011).

Due to the vulnerability of the vegetable to the attack of the deleterious agent and to the increase of the use of agrochemicals in the management of the crops, new management techniques have been adopted to facilitate the diagnosis and control of the PNC in the field. From this perspective, the biological control of plants (biocontrol) arises alternately by the use of biological agents (fungi, viruses, bacteria and nematodes) in the phytosanitary management of diseases, generating less impact on the environment, maintaining the balance of agricultural systems and agrobiodiversity. Thus, endophytic bacteria has been reported in previous research as plant growth promoters (SILVA et al., 2015; SILVA et al., 2019; ARAÚJO et al., 2019).

Therefore, the objective of this study was to evaluate different bacterial endophytes in the semi-arid region of the State of Alagoas, Northeastern Brazil, in the promotion of resistance to PNC in cabbage plants.

2. MATERIAL AND METHODS

2.1 EXPERIMENTAL ÁREA

The experiments were conducted in a laboratory and greenhouse belonging to the Laboratory of Microbiology of the Agricultural Sciences Center of the Federal University of Alagoas, Brazil, at the geographical coordinates 9º 27’57”S, 34º 50’ 1” W longitude and 127 m altitude, located in coastal tablelands. The climate of the region, according to the Köppen classification is type A, that is, tropical hot and humid, with dry season in winter and annual rainfall of 1,630 mm, average annual temperature of 24 ºC and relative humidity of 79%.
2.2 BACTERIAL ENDOPHYTES

The bacterial endophytes used in this study are preserved in the Microorganisms Collection of the Laboratory of Agriculture Microbiology of The Center of Agrarian Sciences of the Federal University of Alagoas. The endophytes were selected in according to his hability of solubilize inorganic phosphate in previous study by Silva et al. (2018). All the bacteria endophytes (ISO31, ISO33, ISO34, ISO48 e ISO51) are from the Bacillus genre.

2.3 IN VITRO EVALUATION OF RESEISTENCE INDUCTION TO XCC

For the production of the seedlings, cabbage leaf (99.9% purity) seeds previously disinfected in sodium hypochlorite solution (10%) were used by the one-minute immersion technique and double washing with distilled water, which was carried out in tray of 200 cells containing substrate (soil and humus 1:1) at 0.5 cm depth of the tray surface.

After the emergence of the first true leaves, the seedlings were transplanted into 500 ml pots filled with autoclaved and non-autoclaved soil (pH in water of 6.2, P: 80 mg/dm³, K: 113 mg/dm³, Ca: 4.55 cmol/dm³, Mg: 2.55 cmol/dm³, Al: 0.01 cmol/dm³, H+Al: 2.70 cmol/dm³, effective CEC: 7.40 cmol/dm³, Saturation of Ca: 45.1%, saturation of Mg: 25.3% and saturation of K: 2.9%). After this, was carried out the inoculation of the bacterial endophytes with aid of a syringe. In each pot was inoculated 3ml of the bacterial solution in rhizospheric region of the plants.

At 30 days after transplanting, 0.2 ml of bacterial solution of Xcc was inoculated, with the aid of a disposable syringe. For this procedure, a random leaf was chosen in each plant, puncturing its main vein and injecting the solution into the tissues of the leaf, consequently causing a wound that allows the penetration and the beginning of the colonization and infection of the Xcc in the cells of the host.

During the incubation period of the pathogen, the development of the symptoms caused by the BRC in the inoculated plants was observed. At the end of seven days and one month after the onset of infection (30 days), the severity of the damage was evaluated by a proposal diagrammatic scale ranging from 0 to 5, being: 0 = absence of symptoms; 1 = start of spot in the area of application; 2 = yellowing spot; 3 = brown or dark brown spot; 4 = necrotic spot; 5 = complete necrosis or leaf death.

2.4 BIOMETRY OF THE PLANTS

At the same time, biometry of characteristics of agronomic interest, such as the number of leaves, plant height (cm), root system length (cm), collar diameter (mm), fresh area and root mass (g) and leaf area (cm²) was carried out.
Subsequently, the samples were conditioned in paper bags and kept in a forced circulation oven at 65 °C for 48 hours to dry. After the drying process, the samples were weighed on a precision scale to determine the dry matter (g).

2.5 DETERMINATION OF TOTAL CLOROPHILL CONTENT

Finally, the determination of the chlorophyll content was carried out according to methodology described by Arnon (1949), with the removal of a leaf disc of approximately 9 mm of each sample (plant), totaling three replicates per treatment. Subsequently, the disks were macerated with 1 ml of acetone (80% v/v). The extract was filtered and read the optical density (OD) in a spectrophotometer (Biospectro Model SP-22) at 645 and 663 nm, and then determining the total chlorophyll concentration by means of the formula (Conc.CI = 20.2 A645nm + 8.02 A663nm), the results being expressed in μg.g⁻¹ leaf.

2.6 EXPERIMENTAL DESIGN AND STATISTICS ANALYSIS

The experimental design was completely randomized, with two treatments for each bacterial isolate evaluated, two positive controls (plants inoculated with Xcc) and two negative (plants without the inoculation of Xcc), constituting a total of 14 treatments with three replicates (T1 = single autoclaved soil + Xcc + ISO31; T2 = autoclaved soil + Xcc + ISO33; T3 = autoclaved soil + Xcc + ISO34; T4 = autoclaved soil + Xcc + ISO48; T5 = non-autoclaved soil + Xcc + ISO31; T6 = non-autoclaved soil + Xcc + ISO33; T7 = non-autoclaved soil + Xcc + ISO34; T8 = non-autoclaved soil + Xcc + ISO48; T9 = non-autoclaved soil + Xcc; T10 = non-autoclaved soil - Xcc; T11 = autoclaved soil - Xcc; T12 = non-autoclaved soil - Xcc; T13 = autoclaved soil - Xcc; T14 = autoclaved soil - Xcc), resulting in 42 plots.

The data were submitted to analysis of variance (ANOVA) and compared by the Scott-Knott test (p≤0.05) by the Sisvar Software (FERREIRA, 2014).

3 RESULTS AND DISCUSSION

The resistance to symptoms caused by the PNC (Xcc) was evaluated by the lesion comparison method according to the diagrammatic scale of damages of the causal agent of the disease (Figure 1), showing that the population of cabbage leaf presented yellowish spots throughout the foliar area to necrotic spots that directly compromise the photosynthetic performance of these plants.
Figure 1 – Diagramatic scale of damage caused by Xcc in cabbage leaf plants, where: A = absence of symptoms; B = initial rot; C = yellowing; D = brown spot; E = necrosis.

After completing 30 days of infection, it was possible to observe the progress of PNC in cabbage plants compared to the first evaluation performed, motivated by the increase in the severity of the lesion stage, to the detriment of the increase in incubation time, all treatments being diagnosed with at least a symptom except the negative controls (T12 and T14) (Figure 2).

Figure 2 – Evolution of the lesions acasioned by Xcc in cabbage leaf plants.

During the incubation period there were no mortality (complete necrosis or leaf death), in which the disease was less severe even in plants inoculated exclusively with the phytopathogen (T11 and T13), without the presence of growth promoting bacteria (BPCV), a behavior different from that found by SILVA et al. (2018), where 100% of the leaves treated with the phytopathogen Xcc underwent drastic phenotypic changes such as necrosis and subsequent tipping off.

One of the factors that may prevent the use of bacterial strains in resistance promotion evaluations is the infectious inoculum life, since old cultures may lose the ability to cause diseases (virulence) over the course of storage time (SOLA et al., 2012).

The significant difference of the negative controls (T12 and T14) in relation to the other treatments (endophytic and positive) could be observed did not differ, however, the numerical differences can demonstrate the behavior of each isolate against the Xcc colonization.

Considering the different soil conditions, all bacterial endophytes obtained lesions lower than the positive control (T13) in autoclaved soil in the initial infection, which demonstrates the full cellular development and the efficiency in the synthesis of mechanisms to promote resistance to leaf symptoms of BRC by bacterial endophytes, without the interference of native antagonist microorganisms.
On the other hand, the isolates ISO31, ISO33 and ISO51 showed tolerance to biotic (soil microbiota) and abiotic agents (autoclaved and non-autoclaved) a factor that does not cause interference in the performance of endophytic bacteria in *in vivo* application.

For the definition of the most effective isolates in the promotion of resistance to BRC, it is necessary besides the observation of the progression of the foliar symptoms, the analysis of the agronomic characteristics of production, that are also directly interfered by the action of the Xcc in the plant tissues, being able to entail losses productivity and phytosanitary problems in rural properties.

The study of the morphological responses in adverse conditions of the habitual (nutritional, water and sanitary) appropriates as a tool for the understanding of the vegetal structural behavior (aerial and subterraneous), being in this work, evaluated besides the resistance of the cabbage plants to BRC, the capacity of plant growth promotion (production variables) by bacterial endophytes against the activity of the etiological agent of the disease (Xcc). The bacterial performance in the biometric parameters is found in table 2, distributed according to the production characteristics evaluated.

It was verified by means of the analysis of variance that there was no significant difference between the bacterial isolates regarding the number of leaves, where, despite the statistical similarity, ISO48 presented higher average when compared to the other treatments, as evidenced in Table 1.

| Bacterial isolate | LN (cm) | APL (cm) | RL (cm) | CD (mm) | LA (cm²) |
|-------------------|---------|----------|---------|---------|---------|
| ISO31             | 8,50 a* | 26,85 a  | 17,88 a | 3,20 a  | 240,00 a|
| ISO33             | 10,16 a | 28,08 a  | 18,38 a | 2,70 a  | 230,50 a|
| ISO34             | 9,33 a  | 28,23 a  | 19,15 a | 3,36 a  | 242,00 a|
| ISO48             | 10,33 a | 26,36 a  | 18,15 a | 3,16 a  | 248,00 a|
| ISO51             | 9,16 a  | 27,28 a  | 18,48 a | 3,31 a  | 232,33 a|
| Control (+)       | 10,16 a | 25,68 a  | 19,30 a | 3,43 a  | 234,16 a|
| Control (-)       | 10,91 a | 27,08 a  | 17,81 a | 2,85 a  | 219,41 a|
|                   | FMAP (g) | FMRS (g) | DMAP (g) | DMRS (g) | TC (μg.g⁻¹) |
| ISO31             | 9,07 a  | 7,74 a   | 1,35 a  | 4,52 a  | 30,43 d  |
| ISO33             | 9,24 a  | 6,78 a   | 1,28 a  | 3,55 a  | 33,20 c  |
| ISO34             | 10,07 a | 9,71 a   | 1,47 a  | 4,80 a  | 35,85 b  |
| ISO48             | 9,60 a  | 7,76 a   | 1,30 a  | 3,74 a  | 38,74 a  |
Table 1 – Biometric parameters of cabbage leaf plants inoculated with endophytic bacteria.

|        | ISO51 | Control (+) | Control (-) |
|--------|-------|-------------|-------------|
| LF     | 9.97 a| 9.75 a      | 8.66 a      |
| APL    | 10.80 a| 7.92 a      | 9.05 a      |
| RL     | 1.43 a | 1.39 a      | 1.11 a      |
| CD     | 5.02 a | 3.35 a      | 4.05 a      |
| LA     | 34.36 b| 32.00 c     | 29.34 d     |
| FMAP   |       |             |             |
| FMRS   |       |             |             |
| DMAP   |       |             |             |
| DMRS   |       |             |             |
| TC     |       |             |             |

*Means followed by the same letter do not differ by Skott-Knott test (p≤0.05). LF: leaf number; APL: aerial part length; RL: root length; CD: collar diameter; LA: leaf area; FMAP: fresh matter of aerial part; FMRS: fresh matter of root system; DMAP: dry matter of aerial part; DMRS: dry matter of root system; TC: total chlorophyll.

Leaves number it is a specific variable has a high importance for the cultivation of kale, since the commercialization of the cultivar is given by the sale of these leaves, in which, the higher the production, the higher the remuneration for the family producer and for the vegetable production chain.

The plants height did not differ statistically (p≤0.05), however, the increase of the endophytic bacteria in the aid of this characteristic is observed, unlike the observed in the evaluation of the number of leaves.

Of the five bacterial isolates evaluated, ISO34, ISO33 and ISO51 showed a higher increase in the promotion of aerial part growth in *B. oleracea* plants, unlike ISO31 and ISO48, which expressed lower weight in comparison with the negative control (without the inoculation of bacterial endophytes).

The size of the plant is an important variable for the establishment of agricultural crops, mainly in field conditions, since the irregular growth of the stand can cause differences in the production pattern. Higher plants have advantages over those with smaller stature, as they can capture more solar energy for their photosynthetic reactions, essential for the normal functioning of plant metabolism.

The root system is responsible for the establishment of plants in field. Further the soil fixation, roots play a role of water absorption and nutrient and essential mineral uptake. Regarding the length of the root system, no statistically significant differences were found between endophytic bacterial isolates (p≤0.05), according to the results described in Table 1.

It was verified by means by the Scott-Knott test (p≤0.05) that there was no significant statistical difference for the variable DC among the evaluated treatments, indicating its independent development of the bacterial increment.

According to table 2, ISO34, ISO51 and ISO31 were classified as the best promoters of increase of the diameter of the colla, however, ISO33 expressed increment lower to the negative control, assuming its direct interference in the increase of this characteristic in the cabbage crop.
The increase of the basal diameter by means of bacterial endophytes of xerophytes can be understood as an adaptive evolutionary expression, since this group of plants needed to increase the diameter of its stem to increase the hydric reserve space, in addition, a more developed colla provides the highest abstraction of water and nutrients, essential for survival in its natural habitat (semiarid regions).

The measurement of the colla is one of the main morphological parameters for the evaluation of the growth of crop species, being the same as the meeting point of the root with the stem. These characteristic can indicate whether there was or not the plants stapling.

Although not statistically different from the other treatments, ISO48 and ISO34 showed the highest averages among the bacterial isolates evaluated, with a difference of 14.50 cm² of leaf area in relation to ISO 33, which obtained the lowest mean (Table 1), which assumes the success of these isolates in the evaluation of fresh matter (vegetal biomass).

Unlike the adaptive mechanism of increase of the colla diameter, the xerphiles over the years tended the strategy of reduction of the evaporative surface (leaf area and stomata) in response to the water deficit, inhibiting the cellular expansion and the leaf development (Santos et al. 2013), which differs from the results found in this study, since the endophytic bacteria collaborated in the promotion of the increase of the leaf area of the cabbage plants.

The endophytic bacteria provided a significant increase in cabbage FMAP, as observed in the LAP and FA evaluations, which shows the limitation of the cabbage plants in the promotion of this variable without the external assistance of the VGPB (Vegetal Growth Promotion Bacteria), and the inoculation of these promoters in the field an effective alternative to increase crop productivity, contributing to the strengthening of sustainable farming practices.

To the detriment of the correlation between FMAP and FA, it is necessary to observe the results obtained in both variables, because plants that have larger leaves tend to present higher aerial part weight to plants with less developed FA.

Thus, isolate 48 expressed the highest mean of AP among the bacterial endophytes evaluated, which would also present the best performance of FMAP, however, ISO34 and ISO51 exhibited the highest fresh matter means, the latter being the holder of the second lowest average in promoting leaf area increase.

For the correct evaluation of the root development it is necessary also the inference regarding the root length. A certain plant may exhibit an elongated root system, however, with low fresh matter, which presupposes the need for root extension to the deeper layers of the soil as a mechanism to facilitate the absorption of nutrients in the more superficial layers, making the root system less developed and with its establishment compromised. In other cases, a plant may not emit many roots,
but present a broad distribution in the soil, an aspect that demonstrates the good nutritional availability of the soil or the presence of growth promoting mechanisms (nutrient supply by the activity of soil microorganisms), corroborating for a good fixation and architecture of the plant population.

As in the variable RL, endophytes ISO51 and ISO34 stood out in front of the other bacterial treatments by presenting the highest mean, which confirms the significant increase in the promotion of root development by both isolates.

Therefore, ISO51 and ISO34 have the capacity to provide the deepening of the system to assist in the water assimilation in the soil and the effective supply of essential nutrients, due to the good development of the root biomass, an indication of root colonization by the bacteria.

The phytopathogen Xcc through the release of extracellular enzymes causes the degradation of cell wall components, such as cellulose, hemicellulose, lignin, among others (ANDRADE et al., 2005), and in this work the induction of resistance promoted by ISO34 and ISO51 to the structural damage caused by BRC in plant cells, as the cabbage plants inoculated with these endophytes did not interfere with the accumulation of FMAP, obtaining an average higher than control (positive control) treatments.

However, the other bacterial isolates (ISO31, ISO33 and ISO48) were not successful in promoting resistance to dry matter deterioration, showing susceptibility to the action of Xcc in this variable.

Such as the length and the biomass, the dry matter of the root system is an indicator of the good morphological development of the roots. Based on this analysis it is possible to observe the microbial aid in root formation, such as the synthesis of coating, meristematic and vascular tissues, promoting their full growth and helping the plant to remain well nourished until its harvest.

The endophytic bacteria ISO51, ISO34 and ISO31 expressed a satisfactory result in the variable DMRS, presenting dry root weight higher than the positive and negative controls, as shown in table 1.

Considering the different soil conditions, treatments on sterilized substrates obtained better performance in the majority of evaluated variables than those grown in non-sterilized soil, according to the data described in table 2.

Table 2 – Biometric parameters of cabbage leaf inoculated with endophytic bacteria in function of soil conditions.

| Soil     | LN (cm) | APL (cm) | RL (cm) | CD (mm) | LA (cm²) |
|----------|---------|----------|---------|---------|----------|
| Autoclaved | 9,45 a | 27,58 a | 17,83 a | 3,44 a | 249,78 a |
| Natural   | 10,14 a | 26,58 a | 19,06 a | 2,85 b | 220,61 b |
It is observed that the use of autoclaved soil for cabbage leaf cultivation has resulted in the potentiation of the biometric parameters of the plants, thus increased by the endophytic bacteria in the variables under sterilized soil conditions, increasing their potential for promotion of plant growth, unlike samples grown on natural soil, which may suffer from some interference with the microbiota or other native organisms or external agents, inhibiting plant growth characteristics from plant growth promoting bacteria.

In figure 3, it is possible to visualize the different shades of green that characterize the concentration of total chlorophyll pigments by the treatments, in which, the darker the extract appears, the concentration of total chlorophylls in the solution, which should be measured by absorbance reading in spectrophotometer.

*Means followed by the same letter do not differ by Skott-Knott test (p≤0.05). LF: leaf number; APL: aerial part length; RL: root length; CD: collar diameter; LA: leaf area; FMAP: fresh matter of aerial part; FMRS: fresh matter of root system; DMAP: dry matter of aerial part; DMRS: dry matter of root system; TC: total chlorophyll.

|      | FMAP (g) | FMRS (g) | DMAP (g) | DMRS (g) | TC (μg.g⁻¹) |
|------|----------|----------|----------|----------|-------------|
| Autoclaved | 10,27 a  | 9,35 a   | 1,53 a   | 4,39 a   | 32,19 b     |
| Natural  | 8,70 b   | 7,72 a   | 1,13 b   | 3,90 a   | 34,64 a     |

Figura 4 – Different contents of total chlorophyll in cabbage leaf plants inoculated with endophytic bacteria. A: Autoclaved soil + Xcc + ISO31; B: autoclaved soil + Xcc + ISO33; C: autoclaved soil + Xcc + ISO34; D: autoclaved soil + Xcc + ISO48; E: autoclaved soil + Xcc + ISO51; F: non-autoclaved soil + Xcc + ISO31; G: non-autoclaved soil + Xcc + ISO33; H: non-autoclaved soil + Xcc + ISO34; I: non-autoclaved + Xcc + ISO48; J: non-autoclaved soil + Xcc
The infection of the etiological agent Xcc causes, besides the destruction of the photosynthetic tissues, the increase of the respiratory rate due to the alteration of the water potential, consequently causing the reduction of the photosynthesis and the total chlorophyll content in the plant cells (DALIO; PASCHOLATI, 2018). This reduction in the concentration of chlorophyll pigments was observed in treatment 13 (autoclaved soil + Xcc), which stopped the worst average in the variable, and in cases of greater severity, the rate of photosynthesis was reduced by up to 75%.

4. CONCLUSIONS

Except ISO31, all the VGPB’s evaluated in this work provided a significant increase in the photosynthetic capacity of the cabbage plants, thus demonstrating the efficiency of the endophytic microorganisms in promoting the plant growth and induce resistence against X. campestris pv. campestris in vivo.

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