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RESEARCH ARTICLE

# Effect of salicylic acid on cowpea seedlings under saline stress

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## Abstract

The aim of this work was applying salicylic acid (SA) in cowpea seedlings under saline stress. The experiment took place in the seed laboratory of the Universidade Federal Rural da Amazônia with a completely randomized experimental design in a 2 x 2 x 3 factorial scheme, with 2 bean cultivars (Canapu and Pingo-de-ouro), 2 levels of salicylic acid (0 and 0.50 mM) and 3 salt stress levels (0, 25, 50 mM). The seeds were previously soaked in salicylic acid (0 and 0.50 mM) for a period of 12 hrs and then placed in germitest paper rolls for treatments with NaCl (0, 25, 50 mM) for a period of 12 days at room temperature constant 27 °C. There was a significant effect of cultivars, AS dose and NaCl concentrations and their interactions on most of the analyzed variables. Root and leaf proline concentrations were higher in pingo-de-ouro cultivar, Canapu cultivar had better performance in biomass accumulation. Salicylic acid reduced proteins in the leaves by 13.33%, while in the root there was an increase of 12.61%, ammonium concentrations reduced in the roots by 11.9%. When applied to salinity (25 and 50 mM), there was an increase of proteins in the leaves 40.83% and 27.48% respectively, and a reduction of amino acids of 30.24 and 25.24% in NaCl dosages (25 and 50 mM) respectively. Salinity reduced biomass accumulation and interfered with cellular solute production. However, the application of salicylic acid promoted salt stress tolerance in Canapu cultivar.

## Keywords

Aminoacids; proline; glycine-bethayne; salinity; carbohydrates

## Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is an herbaceous and annual plant belonging to the genus *Vigna*, family Fabaceae (1,2). It is one of the most adapted, versatile and nutritious among the cultivated legume species, endowed with high protein content and low production cost (3-5).

Cultivated predominantly in the North and Northeast regions of Brazil, it has great importance as a source of employment and income (6), and constitutes one of the main components of human food in these regions (7). On the world stage, production is around 5589216 t, with the African continent representing 95.68% of world production, followed by the Asian and American continents. (8).

Although cowpea is considered a tropical crop with wide adaptation to the most diverse environments, the yield is still considered low and salinity is one of the main causes (9-11), since the arid and semi-arid regions where it is widely cultivated are characterized by low rainfall and high evaporative demand, high levels of salts in part of their water sources and inadequate management of irrigation and drainage, promoting soil salinization (12,13).

Salinity affects several aspects of plant physiology and biochemistry by reducing water absorption through the specific effect of ions or their excess in the plant (14). Studies point to changes in cellular metabolism and the functioning of some enzymes, disturbance of ionic balance, nutritional imbalance, reduction of osmotic potential, reduction of nutrient assimilation, reduction in growth, reduction in photosynthesis and production of reactive oxygen species (15-17).

Cowpea is classified as moderately tolerant of irrigation with saline water, with levels around 3.3dS m-1, without significant losses in production, varying between genotypes and the ionic composition of the medium (15, 18). However, it is of paramount importance to develop management methods that help cowpea in the germination and establishment stage to tolerate saline stress levels (19).

Salicylic acid (SA) is an endogenous plant regulator that has a signaling function for the induction of tolerance and defense mechanisms against different types of abiotic stresses (20), such as saline stress. Studies have demonstrated the ability of SA to act as an inducer of tolerance proteins in plants under saline stress, promoting changes in cell metabolism, activating mechanisms of tolerance and plant survival (21). However, the effects of SA are dependent on factors such as concentration, plant species, method and application dose (22). In this sense, we seek to evaluate of SA application effects on the ability of cowpea (*Vigna unguiculata* (L.) Walp) seedlings to saline stress.

## Materials and Methods

### *Experiment and plant materia*

The experiment was carried out in the seed laboratory of the Instituto de Ciências Agrárias, located at the Universidade Federal Rural da Amazônia (UFRA), Belém – PA, with geographic coordinates 27' 21'' S, 30' 16'' W and an average altitude of 10 m. The biochemical and physiological analyzes were carried out at the Laboratory of Biodiversity Studies in Higher Plants (EBPS).

The experimental design was completely randomized in a 2 x 2 x 3 factorial scheme, with 2cultivars, combined with 2levels of SA (0 and 0.50 mM) and 3levels of salt stress (0, 25, 50 mM), totalling 12 treatments and consisting of 4replications of 25 seeds.

The plant material used was the cultivars Pingo-de-ouro and Canapu, species of cowpea (*Vigna unguiculata* (L.) Walp), classified as sensitive and moderately sensitive to salt stress respectively (23).

Cowpea seeds were previously selected based on the uniformity among them, disregarding those with deformities. After selection, they were subjected to asepsis in 98% alcohol for 30s, then in 2% hypochlorite for a period of 1 min and then washed 3 times with distilled water. The statistical method used was that of Scott Knott.

### *Pre-treatment with pure salicylic acid (SA) and exposure of seeds to NaCl*

The acid was dissolved in distilled water to a concentration of 0.50 mM. The seeds were placed on germitest paper in a gerbox and soaked for a period of 12hrs, in addition to the control treatment in which the seeds were soaked in distilled water for the same period. Subsequently, the seeds were sown in germitest paper rolls with fractions of 25 seeds for treatments with NaCl (0, 25, 50 mM) and later packed in transparent plastic bags. Each roll was placed in a plastic cup to keep it vertical, with normal seedling growth. The experiment was carried out for a period of 12 days at a constant temperature of 27 °C.

In this experiment, the following variables were evaluated: shoot dry mass (SDM), root dry mass (RDM), the results were expressed in g; free ammonium concentration (NH4+) (Weatherburn *et al*., 1967); total soluble protein (TSP) (Bradford *et al*., 1976); glycine-betaine (GB) (Grieve; Grattan *et al*., 1983); proline (PRO) (Bates *et al*., 1973); total soluble amino acids (TSA) (Peoples *et al*., 1989) and total soluble carbohydrates (TSC) (Dubois, 1956). The results were submitted to analysis of variance (ANOVA) and when there was a significant difference, the ScottKnott test was applied (P<0.05).

### *Sampling and analysis of biomass accumulation*

Seedlings were collected on the 12th day after sowing at 1:00 pm. The seedlings were separated into root and shoot and the material was taken to a forced air ventilation oven at 65 °C for 48 hrs (until they reached constant weight). After drying, the dry mass of the shoot and root was determined using a scale. Subsequently, the dry material was ground in a mill until obtaining a fine powder and properly stored in plastic bags until its use in biochemical evaluations.

### *Biochemical evaluations*

Free ammonium (CAL): It was determined using the method according to Weatherburn, 1967, the results were expressed in mmol of $NH_4^+ Kg^{-1}$ of DM; Total Soluble Proteins (PST): Soluble protein contents were determined by the method described by Bradford, 1976. The contents of soluble proteins are expressed in mg protein $g^{-1}$ DM of tissue; Proline (PRO): results were expressed in µmol Proline g-1 DM. For this, we used the method described by Bates *et al*.,1973; Glycine-betaine (GB): results were expressed as µmol of glycine-betaine $g^{-1}$ DM. The method used was according to Grieve and Grattan, 1983; Total Soluble Amino Acids (TSA): The method used was according to Peoples *et al*., 1989, results were expressed in µmol of AA g-1 MS; Total Soluble Carbohydrates (TSC) The standard method was used andthe results were expressed in mmold and GLUg.

## Results and discussion

There was a significant effect of cultivars, SA dose and NaCl concentrations and their interactions on most biochemical and biomass accumulation variables.

### *Cultivars*

For cultivar data, a significant difference was observed for proline concentrations (leaf and root) and shoot dry mass (Table 1). The Pingo-de-ouro cultivar presented the highest means for the root and leaf proline variables. While for the variable SDM, the cultivar Canapu had better performance in the accumulation of biomass.

**Table 1**. Proline concentrations.

| Cultivar | PROf (μmol Pro.g$^{-1}$ DM) | PROr (μmol Pro.g$^{-1}$ DM) | SDM (g) |
|---|---|---|---|
| Canapu | 10.83 b | 4.89 b | 1.17 a |
| Pingo d'ouro | 14.57 a | 6.34 a | 0.67 b |

**PROf** (leaf), **PROr** (root) and leaf dry matter (**SDM**) of cowpea seedlings. Different letters indicate significant differences by the Scott-Knott test (P<0.05).

High levels of proline have been associated with the protection of cellular structures during dehydration in seeds and it is involved in the primary metabolism of seedling development, under stress conditions, proline levels can increase up to one hundred times more than under normal conditions, being a way of indicating plants with higher levels of resistance (24-26). This may suggest that Pingo-de-ouro presents better performance in the primary development of seedlings.

Seedlings with greater accumulation of biomass usually come from seeds with high physiological quality, which provide greater translocation of reserves from cotyledons or endosperm to the embryonic axis during the germination process, making it possible to obtain more vigorous seedlings (27). The data found in this work diverge from Sá *et al*. (23), who found that cv. Pingo-de-ouro showed a higher average in biomass accumulation compared to Canapu, suggesting a low physiological quality of the seed even after the selection was performed.

### *Salicylic acid application*

The application of salicylic acid affected the content of total soluble proteins (leaf and root), free ammonium and root dry matter (Table 2). In the present study, it was observed that the application of salicylic acid (50 mM) caused an increase of 12.61% in root proteins compared to the control treatment.

The increase on the root protein concentration is presumably a better mechanism of plant resistance (28), due to SA promoting structure, maintenance and inhibition of protein degradation (20,29).

Regarding the effect of ammonium concentration in roots, SA (50 mM) promoted an increase of 27.80% when compared to the control treatment. High levels of free ammonium has accumulated under stress conditions, and it is positively correlated with the accumulation of many free amino acids that are related to plant tolerance signalling (30).

**Table 2**.Total soluble protein concentrations.

| Acid (mM) | PSTf (mg protein.g$^{1}$MS) | PSTr (mg protein.g$^{1}$MS) | CALr (mmol de NH4 Kg$^{1}$ de MS) | RDM (g) |
|---|---|---|---|---|
| 0 | 3.45 a | 3.25 b | 126.36 b | 0.42 a |
| 50 | 2.99 b | 3.66 a | 161.49 a | 0.37 b |

**PSTf** (leaf), **PSTr** (root); free ammonium – **CALr** (root); root dry matter (**RDM**) of cowpea seedlings in relation to salicylic acid application (0 and 50 mM). Different letters indicate significant differences by the Scott-Knott test (P<0.05)

In relation to root dry matter, application of SA (50 mM) promoted a reduction of 11.90%. Different results were found by other authors. When applying SA in *Foeniculum vulgare* plants at doses of 0.0, 0.5 and 1.0 mM it promoted an increase in RDM (31)and found significant increases in the RDM of plants treated with 100 mg L$^{-1}$ and 200 mg L$^{-1}$ of SA (32).

### *Saline Stress (NaCl)*

For saline stress, there was a significant difference for the concentrations of proteins (leaf and root), total soluble amino acids (root), glycine-betaine (root) and soluble carbohydrates (leaf) (Table 3). The application of NaCl (25 and 50 mM) increased the concentration of total soluble proteins in leaves by 40.83% and 27.48% when compared to control plants. Suggesting that the accumulation of these organic compounds occurs in the cytosol where it promotes osmotic adjustment (33, 34) and also serves as an energy reserve and source of carbon skeletons for the formation of seedling tissues under stress conditions (28).

**Table 3**.Total soluble protein concentrations.

| NaCl (mM) | PSTf (mg protein.g-1DM) | PSTr (mg protein.g-1DM) | GBr (mg glicina-betain.g$^{-1}$ DM) | ASTr (μmol of AA/g DM) | CSTf (mmol de GLUg$^{-1}$) |
|---|---|---|---|---|---|
| 0 | 2.62 b | 3.76 a | 23.93 a | 24.01 a | 3.77 b |
| 25 | 3.69 a | 3.70 a | 19.10 b | 16.75 b | 3.30 b |
| 50 | 3.34 a | 2.90 b | 27.95 a | 17.95 b | 4.48 a |

**PSTf** (leaf), **PSTr** (root); glycine-betaine–**GBr** (root), total soluble amino acids – **ASTr** (root); total soluble carbohydrates – **CSTf** (leaf) in cowpea seedlings in relation to NaCl application (0, 25 and 50 mM). Different letters indicate significant differences by the Scott-Knott test (P<0.05).

Regarding the concentration of total soluble proteins in the roots, there was no statistical difference between the control seedlings and those that received 25 mM of NaCl, suggesting that this level of saline stress did not negatively influence the cowpea cultivars. However, in the seedlings treated with 50 mM of NaCl there was a significant decrease of 22.87% in the concentration of total soluble proteins. Salt stress may have promoted a decrease in protein concentration since protein synthesis is impaired due to the overproduction of reactive oxygen species and there is an increase in proteolysis (35,36).

Regarding the concentration of glycine-betaine (GB) in the root, there was no statistical difference between control and seedlings treated with 50 mM of NaCl, however they were higher than the values found in seedlings submitted to 25 mM of NaCl. Glycine-betaine accumulation is presumed to be involved in the osmotic adjustment and/or osmoprotection of cellular functional macromolecules, the defense of ionic and oxidative stress in tissues under saline stress (37,38).

The concentrations of total soluble amino acids had significant reductions of 30.24 and 25.24% when submitted to saline stress respectively to the treatments of 25 and 50mM of NaCl. There was another study observed this same behavior when evaluating the effects of salt stress on cauliflower plants, in which the amino acid concentration was reduced to a certain level of salinity (39).

For foliar total soluble carbohydrate concentrations there was a significant decrease with the application of 50mM of NaCl of 18.83%, with an average of 4.48 mmol of GLU $g^{-1}$. Similar results were also obtained (39), when they observed a decrease in foliar carbohydrate concentration in cauliflower at 60 days after transplanting as salinity increased. The intriguing result was explained by the changes that salt stress can cause in the processes of carbohydrate production, such as photosynthetic pigments, chlorophyll bloom and photosynthesis.

### *Cultivar X Salicylic acid (SA)*

As for the cultivar X salicylic acid interaction, there was a significant difference for the concentrations of glycine-betaine (leaf and root), total soluble amino acids (leaf) and total soluble carbohydrates (leaf) at 5% ($p<0.05$) (Table 4).

For the glycine-betaine concentrations in the leaves, there was a significant difference between the cultivars without SA application. The Canapu cultivar had a higher average of 23.17 mg glycine-betaine.$g^{-1}$ DM, while the Pingo-de-ouro cultivar had an average of 15.30 mg glycine-betaine.$g^{-1}$ DM. There was no significant difference between treatments with salicylic acid among cultivars.

**Table 4**.Glycine-betaine concentrations.

| Cultivar | Acid (mM) | GBf (mg glicina-betaine.g-1 DM) | GBr (mg glicina-betaine.g-1 DM) | ASTf (µmol de AA/g DM) | CSTf (mmol de GLUg-1) |
|---|---|---|---|---|---|
| Canapu | 0 | 23.17 Aa | 20.39 Ab | 15.60 Ab | 3.85 Aa |
| | 50 | 20.18 Aa | 38.33 Aa | 24.80 Aa | 3.38 Ba |
| Pingo d'ouro | 0 | 15.30 Ba | 17.14 Aa | 20.31 Aa | 3.32 Ab |
| | 50 | 18.89 Aa | 18.78 Ba | 11.67 Bb | 4.86 Aa |

**GBf** (leaf) and **GBr** (root); total soluble amino acids – **ASTf** (leaf); total soluble carbohydrates – **CSTf** (leaf) in relation to cultivar interaction (canapu and pingo-de-ouro) and application of salicylic acid (0 and 50 mM) in cowpea seedlings. Columns with different capital letters between treatments of Cultivar (Canapu and Pingo-de-ouro under the same acid concentration) and lowercase letters between acid treatments (0 and 50mM acid under the same cultivar) indicate significant differences by the Scott-Knott test ($P<0.05$).

Glycine-betaine is a quaternary ammonium compound that has an osmoregulatory and protective function, promoting osmotic adjustment along with other compounds (28). Plant varieties that have a higher concentration of glycine-betaine generally exhibit improved growth and productivity, relative to varieties that do not accumulate (40).

Regarding the concentrations of glycine-betaine in the root, cv. Canapu showed a higher mean of 38.33 mg glycine-betaine.$g^{-1}$ DM compared to the mean of 18.78 mg glycine-betaine.$g^{-1}$ DM of cv. Pingo-de-Ouro under the application of salicylic acid. Salicylic acid can promote a positive effect by inducing an accumulation of GB in the vacuole or in the root cytosol as a protective mechanism and osmotic adjustment (24). It was showed that salicylic acid induced the accumulation of GB in *Vigna radiata* with consequent increase in glutathione, reduction of ethylene and oxidative stress and improvement of photosynthesis (41).

For the amino acid concentrations in the leaf, the application of SA (50 mM) promoted an increase of 42.54% in cv. Canapu, while in cv. Pingo-de-Ouro, a reduction of 58.97%. The possible effects of SA regulators on amino acid accumulation are poorly known and may differ between plants, but the increase in TSA concentration suggests an improvement in the nitrogen content in the leaves of plants when treated with SA (42).

With reference to the concentration of total soluble carbohydrates in the leaves, the application of salicylic acid (50 mM) promoted a significant increase of 31.68% in cv. Pingo de Ouro compared to cv. Canapu. The results obtained are similar to those reported earlier (43), using pre-treatment of 0.5 mM of SA in chickpea seeds, which found a higher concentration of carbohydrates and higher reproductive yield when compared to both inoculation with arbuscular mycorrhizal as the control plants. This behavior was explained by the application of SA to modulate carbohydrate metabolism from the synthesis of sugars to form new cellular constituents as a mechanism to promote plant growth.

### *Cultivar X NaCl*

A significant variable for the cultivar interaction and salt stress in cowpea seedlings was illustrated in Table 5. The cv. Canapu showed higher averages of RDM compared to cv. Gold flecks as salinity increases.

**Table 5**.Interaction of cultivars (Canapu and Pingo-de-ouro) and application of NaCl (0, 25 and 50 mM) in cowpea seedlings on root dry mass.

| Cultivar | NaCl (mM) | RDM (g) |
|---|---|---|
| | 0 | 0.41 Aa |
| Canapu | 25 | 0.46 Aa |
| | 50 | 0.44 Aa |
| | 0 | 0.48 Aa |
| Pingo-de-ouro | 25 | 0.32 Bb |
| | 50 | 0.25 Bb |

Columns with different capital letters between cultivar treatments (Canapu and Pingo-de-ouro under the same acid concentration) and lowercase letters between acid treatments (0 and 50 mM acid under the same cultivar) indicate significant differences by the Scott-Knott test (P<0.05).

Ionic toxicity caused by saline stress can affect the physiological and metabolic processes of embryonic tissue, delaying root elongation (44). In addition, seedling growth and biomass accumulation is compromised due to decreased cell expansion and elongation, stomatal closure and decreased stomatal conductance (12, 30).

Based on this, it can be assumed that cv. Pingo- deouro is more susceptible to saline stress than cv. Canapu, corroborating the data recorded, in a study with seed germination and vigor in cowpea cultivars subjected to saline stress (23).

### GSalicylic acid (SA) X NaCl

For the SA x NaCl interaction, there was a significant difference for the variables proline (root) and total soluble amino acids (leaf) at 5% ($p<0.05$) (Table 6).

Salt stress promoted a significant increase in PROr concentration by 76.04% (25 mM) and 169.46% (50 mM). While the application of salicylic acid under saline stress promoted a significant reduction of 64.23% in the concentration of root proline when compared to non-stressed seedlings.

Under saline stress, osmotic adjustment can be achieved by the accumulation of organic solutes in the cytoplasm, especially proline (22), as it is an osmoprotector, protects plants from stress and acts in response to environmental changes and serve as a biochemical and physiological indicator of the effects of salt stress on cultivated plants (24).

Similar results were recorded in which testing different concentrations of NaCl (0; 25; 50; 75; 100mM) in cassava genotypes verified the increase in proline accumulation from the increase in salt and explained that this synthesis of proline is an indicator biochemistry of salt stress *in vitro* cultivated cassava plants (45).

As for the interaction of SA and NaCl, opposite results were reported, when verifying changes induced by salicylic acid in growth, biochemical attributes and antioxidant enzymatic activity in *Vicia faba* L. seedlings under NaCl toxicity, observed an increase in proline concentration (46). Since generally the application of salicylic acid during saline stress conditions has a promising effect by providing an increase in proline concentrations, which is one of the main compatible solutes that participate in cell protection and maintain the continuous flow of water. (47,48).

### Cultivar X Salicylic Acid (SA) X NaCl

There was a significant effect of the three factors studied (cultivar x SA x NaCl) on foliar concentrations of free ammonium at 5% ($p<0.05$) (Table 7).

Pingo-de-ouro had a higher average of 321.97 mmol of $NH_4^+.Kg^{-1}$ of DM compared to the average of 173.17 mmol of $NH_4^+.Kg^{-1}$ of DM of cv. Canapu subjected to 50mM NaCl (Table 7). During salt stress, high ammonium produc-

**Table 6**.Concentrations of proline.

| Acid (mM) | NaCl (mM) | PROr (µmol Pro.g$^{-1}$ DM) | ASTf (µmol de AA/g DM) |
|---|---|---|---|
| | 0 | 3.34 Ac | 23.11 Aa |
| 0 | 25 | 5.88 Ab | 17.04 Aa |
| | 50 | 9.00 Aa | 13.72 Aa |
| | 0 | 5.07 Aa | 15.36 Aa |
| 50 | 25 | 4.93 Aa | 18.05 Aa |
| | 50 | 5.48 Ba | 21.29 Aa |

**PROr** (root) and total soluble amino acids – **ASTf** (leaf) in relation to the interaction of application of salicylic acid (0 and 50 mM) and NaCl (0, 25 and 50 mM) in cowpea seedlings. Columns with different capital letters between acid treatments (0 and 50 mM under the same NaCl concentration) and lower case letters between NaCl treatments (0, 25 and 50 mM NaCl under the same acid concentration) indicate significant differences by the Scott- knott (P<0.05).

**Table 7**.Free ammonium foliar concentrations in relation to the interaction of salicylic acid application (0 and 50 mM) X cultivars (Canapu and Pingo-de-Ouro) X NaCl (0, 25 and 50 mM) in cowpea seedlings.

| Cultivar | AS (mM) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | | 50 | | |
| | NaCl (mM) | | | | | |
| | 0 | 25 | 50 | 0 | 25 | 50 |
| Canapu | 171.29 Aa | 187.51 Aa | 173.17 Ba | 214.76 Aa | 192.29 Aa | 175.24 Aa |
| Pingo-de-ouro | 176.77 Ab | 203.81 Ab | 321.97 Aa | 221.43 Ab | 266.00 Aa | 96.60 Ac |

Columns with capital letters and rows with different lowercase letters indicate significant differences by the Scott-Knott test (P<0.05).

tion occurs, acting as an efficient signal to activate coordinated responses involved in the regulation of $Na^+$ homeostasis in plants under salt stress, which leads to salt tolerance (49).

With the application of salicylic acid (50 mM) under 50mM of NaCl, there was a significant decrease in the foliar concentration of free ammonium for cv. Golden drop (Table 7). The reduction in the concentration of free ammonium may indicate an improved capacity to use this ion for the production of amino acids and protein synthesis (50). Based on this, it can be assumed that the application of salicylic acid promoted the incorporation of ammonium into organic compounds, avoiding possible toxic effects of ammonium.

## Conclusion

The biomass accumulation and seedling biochemistry of *Vigna unguiculata* (L.) Walp. were negatively affected by the increase in saline concentration, mainly the variables proteins and amino acids. Furthermore, cv. Canapu proved to be more tolerant to saline stress, showing greater accumulation of biomass and thus better performance at a concentration of 50mM NaCl. The 50 mM dose of salicylic acid used promoted an increase in glycine-betaine levels in leaves and roots, in addition to carbohydrate and amino acid concentrations in leaves, demonstrating the interaction of SA with the accumulation of organic solutes. The application of salicylic acid enabled plants to tolerate salt stress in the Pingo de Ouro cultivar, by reducing the concentration of potentially toxic free ammonium, which may indicate that SA promotes an improvement in the ability to use this solute. According to the results collected in this study, it allows inferring some indications in relation to the cowpea culture, the salicylic acid dosage and the salinity level. Regarding culture, canapu can be indicated as more tolerant at a concentration of 50 mM of salt under a dose of 50 mM of SA. However, further studies are required that address other characteristics and other variables in order to have a safe indication for farmers.

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## Authors contributions

DJ and MA carried out the implementation and development of the work. DJ, TM, GA, LS drove formatting. GA, AE and LS corrected the work. CF performed the supervision. GD performed the data analysis. All authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest**: Authors do not have any conflict of interests to declare.

**Ethical issues**: None

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