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Author(s): Alexander Machado Auad and Tiago Teixeira de Resende

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Use of chemical inducers as a resistance trigger in *Brachiaria* grasses and sugarcane

Alexander Machado Auad^{1,*} and Tiago Teixeira de Resende¹

Abstract

Chemical inducers have relevant roles in activating the defense responses of plants to insects. The present research examines the defense mechanisms of *Brachiaria* (Poaceae) grasses and sugarcane cultivars exposed to exogenous of chemical inducers with and without injury due to *Mahanarva spectabilis* (Distant) (Hemiptera: Cercopidae). Plants were subjected to herbivory by spittlebug nymphs and to 3 chemical inducers: salicylic acid, silicic acid, and nitric oxide. Dry matter was calculated at 15 d after induction, and extraction and quantification of phenolic compounds were performed. The phenolic content and percentage of dry matter of the plants was evaluated by analysis of variance, and means were compared by the Scott-Knott test ($P < 0.005$). Changes in dry matter were assessed with regard to interactions between inducers and *Brachiaria* roots and sugarcane shoots. Salicylic acid, silicic acid, and nitric oxide, as well as attack by *M. spectabilis*, triggered alterations in phenolic contents. The highest and lowest concentrations of phenolic compounds were found in the standard cultivar susceptible (cv. Basilik) and resistant to the insect pest (cv. Marandu), respectively. However, the results showed that inducers failed to impact the defense mechanism of *Brachiaria* cultivars and that assessment of total phenolic compounds is not the most adequate approach for measuring resistance in these cultivars. In sugarcane, inducers did not appear to act on defense mechanisms, though the resistant cultivar did exhibit a greater phenolic content. Hence, the use of the above chemical inducers is not useful for triggering resistance in *Bracharia* and sugarcane.

Key Words: Forage; herbivory; induced resistance; phenolic compounds

Resumen

Los inductores químicos tienen un papel relevante en la activación de las respuestas de defensa de las plantas frente a los insectos. La presente investigación examina los mecanismos de defensa de los pastos del género *Brachiaria* (Poaceae) y cultivares de caña de azúcar expuestos a inductores químicos exógenos con y sin lesiones debido a *Mahanarva spectabilis* (Distant) (Hemiptera: Cercopidae). Las plantas fueron sometidas a herbivoría por ninfas de salivitas y por 3 inductores químicos: ácido salicílico, ácido silícico, y óxido nítrico. Se calculó la materia seca a los 15 d después de la inducción y se realizó la extracción y cuantificación de los compuestos fenólicos. Se evaluaron el contenido fenólico y el porcentaje de materia seca de las plantas mediante análisis de varianza y se compararon los medios mediante la prueba de Scott-Knott ($P < 0,005$). Se evaluaron los cambios en la materia seca con respecto a las interacciones entre los inductores y las raíces de *Brachiaria* y los brotes de caña de azúcar. El ácido salicílico, ácido silícico, y óxido nítrico, así como el ataque de *M. spectabilis* provocaron alteraciones en el contenido fenólico. Se encontraron las concentraciones más altas y más bajas de compuestos fenólicos en el cultivar estándar susceptible (cv. Basilik) y resistente (cv. Marandu), respectivamente, a la plaga insecto. Sin embargo, los resultados mostraron que los inductores no lograron afectar el mecanismo de defensa de los cultivares de *Brachiaria* y que la evaluación de los compuestos fenólicos totales no es el enfoque más adecuado para medir la resistencia en estos cultivares. En la caña de azúcar, los inductores no parecen actuar sobre los mecanismos de defensa, aunque el cultivar resistente sí exhibió un mayor contenido fenólico. Por lo tanto, el uso de los inductores químicos mencionadas no es factible para provocar la resistencia en *Bracharia* y caña de azúcar.

Palabras Clave: Forraje; herbivoría; resistencia inducida; compuestos fenólicos

Spittlebugs (Hemiptera: Cercopidae) are the principal pests in forage grasses in tropical America (Valério & Nakano 1988). Several species of spittlebug are found in Brazil; in particular, those of the genus *Mahanarva* (Hemiptera: Cercopidae) cause great damage to plants (Auad et al. 2007; Garcia et al. 2007). Damage to pasture land caused by the yearly attack of spittlebugs is a recurrent issue, and Cercopidae drastically reduce the production and pasture quality of susceptible forages (Valério 1995). Indeed, it has been estimated that losses caused by these insects range between US\$ 840 million to 2.1 billion per yr worldwide (Thompson 2004).

Spittlebug control by chemical methods is impaired by economic and environmental restrictions because pastures are considered of low economic value per unit area and because the application of pesticides

in large areas can reduce wildlife, predators, and parasitoids. Hence, alternative management measures are required. The principal approaches consist of environmentally friendly alternative control measures such as antibiosis mechanisms (Valério et al. 1997; Cardona et al. 1999; Auad et al. 2007). For example, the diversification of pastures by including spittlebug-resistant grasses is a promising strategy to reduce damage by these insects. However, because there is a long time span between the discovery of a resistant forage and the launching of a cultivar, the induction of resistance via the application of chemical inducers may be justified.

Induced resistance is the product of the activation of unique plant defense pathways, which may cause changes in the composition and quantity of secondary metabolites and defense proteins (Vendramin

¹Embrapa Dairy Cattle Research Center, Cep. 36038-330, Juiz de Fora, Minas Gerais, Brazil; E-mails: alexander.auad@embrapa.br (A. M. A.); tiago.resende@embrapa.br (T. T. R.)

*Corresponding author; E-mail: alexander.auad@embrapa.br

& França 2006). Nonetheless, the production of these compounds by a plant has an associated fitness cost, and the equilibrium between constitutive and induced defense mechanisms may be altered by both genotype and environment (Gatehouse 2002). With regard to constitutive defense, salicylic acid is considered to function as a hormone-like endogenous regulator, influencing a range of diverse processes in plants. Some studies have reported that exogenous salicylic acid can ameliorate the harmful effects of drought stress in plants (Simaei et al. 2011). When applied exogenously, silicon (as silicic acid) will accumulate and polymerize on the walls of leaf cells to generate a mechanical barrier that impairs insect attack (Yoshida 1981). Silicic acid also induces chemical defenses (Gomes et al. 2008; Ranger et al. 2009) and may negatively affect the performance of sap-sucking insects (Ferreira et al. 2011; Korndorfer et al. 2011). Nitric oxide is involved in plant physiological processes and affects plant growth, development, and responses to biotic and abiotic stresses (Beligni & Lamattina 2001; Wilson et al. 2008), and exogenous application of this gas confers tolerance to various abiotic stresses in plants by enhancing both enzymatic and non-enzymatic antioxidant defense systems (Xu et al. 2010).

Overall, silicic acid, salicylic acid, and nitric oxide are viable alternatives because they have an important role in the activation of plant defense responses to stress (Klessig et al. 2000; Simaei et al. 2011). Accordingly, we hypothesized that these inducers can be applied exogenously to increase the production of secondary metabolites (phenolics) in plant tissues, eliciting resistance in *Brachiaria* grasses and sugarcane. Therefore, current study evaluates the feasibility of using salicylic acid, silicic acid, and nitric oxide as inducers of resistance in *Brachiaria* and sugarcane on the spittlebug *Mahanarva spectabilis* (Distant) (Hemiptera: Cercopidae).

Materials and Methods

Four *Brachiaria* (Poaceae) cultivars were evaluated: *B. brizantha* Stapf (cv. Marandu) and *B. decumbens* Stapf (cv. Basilisk), which are resistant and susceptible standards to spittlebugs, respectively (Miles et al. 2006), and *B. ruziziensis* Germ. & Everard (cv. Ruziziensis) and *B. ruziziensis* (cv. RJE) susceptible to insect pest. The material was obtained from the forage improvement program of Embrapa Gado de Leite, Brazil. *Brachiaria* seedlings were collected from the Embrapa experimental field in Coronel Pacheco, Minas Gerais, and replanted in plastic tubes (3 cm diam × 10 cm height) with commercial pine bark as the substrate. After 20 d, these seedlings were permanently transplanted to plastic pots containing 500 mL of substrate (soil, manure, and sand in the proportions 3:1:1). Sugarcane cultivars RB 73-9735 and RB 83-5486 from the sugarcane improvement program of the Universidade Federal de Viçosa, Brazil, were propagated vegetatively in plastic pots with a capacity of 500 mL.

The experiment was conducted in a greenhouse with a mean temperature of 22 °C (minimum = 11.9, maximum = 35.9 °C) and mean relative humidity (RH) of 85% (minimum = 42.6, maximum = 98.7). These parameters were recorded using a HOBO DATALOGGER (Onset Co., Pocasset, Massachusetts, USA) and transferred to software (Hoboware); the values represent the means during the experimental period.

Salicylic acid (for *Brachiaria*), silicic acid (for sugarcane), and nitric oxide (for both forages) were applied alone or in combination with *M. spectabilis* nymphs. In the case of tests with salicylic acid and silicic acid, 4 mL of a 1% solution was applied per 500 mL pot on the ground surrounding the plants 2 d after the start of the assay. In the case of the nitric oxide test, the shoot of each plant was sprayed with 2 mL of a solution of 0.15 g per L of a nitric oxide donor solution, sodium

nitroprusside, 2 d after the start of the assay, followed by applications at a 3-d interval. The same procedure was followed in the case of plants that were sprayed with distilled water (treatment control).

Spittlebug nymphs were used to verify resistance induction and were collected from the experimental fields of Embrapa Gado de Leite, Juiz de Fora, Minas Gerais, and maintained on elephant grass in a greenhouse. Four fourth and fifth-instar nymphs from the rearing stock were placed in 500-mL pots, which were covered by a lid containing a central hole to allow the passage of shoots; a layer of gauze was placed around the hole to prevent the escape of spittlebugs. The nymphs were kept on the plants for 15 d; dead nymphs or emerged adults were replaced daily to maintain nymph density.

After 15 d with the inducers, the shoots were cut and weighed, and fresh mass was recorded. The roots were washed on a sieve with running water to remove soil and then placed on absorbent paper for 24 h to eliminate excess water; the material was then weighed for fresh mass determination. The shoots and roots were dried in an oven at 55 °C until constant weight was attained, and the dry mass was recorded. The dried material was ground, and each sample was identified and tagged for biochemical analysis. Phenols were extracted, and their levels in plants subjected to different treatments was assessed by the Folin-Denis method using a spectrophotometer at 760 nm (AOAC 1960).

Randomized blocks were employed in a factorial scheme composed of inducers: application of salicylic acid, silicic acid, nitric oxide with or without *M. spectabilis* nymphs; control treatment and cultivars of *Brachiaria* (4 cultivars) with 5 replications; or sugarcane (2 cultivars) with 8 replications per treatment. Total phenolic compounds and the percentage of dry matter of shoot and roots were calculated. Data were analysed by 2-way analysis of variance (ANOVA), and the means were compared using the Scott-Knott test (1974) ($P \leq 0.05$ was considered significant). The analyses were performed using the program SISVAR 5.3 (2010) (Federal University of Lavras, Lavras, Minas Gerais, Brazil).

Results

TOTAL PHENOLIC COMPOUNDS IN *BRACHIARIA* AND SUGARCANE

No significant variation in phenolic compound content among the *Brachiaria* cultivars without inducers (control) was found for shoots ($F = 0.59$; $df = 3$; $P = 0.6194$) or roots ($F = 1.35$; $df = 3$; $P = 0.261$).

Although we observed no significant interaction between the *Brachiaria* cultivars and inducers with regard to phenolic compound content in shoots ($F = 1.14$; $df = 15$; $P = 0.3297$), isolated activity of these factors (cultivars and inducers) was observed. For example, the phenolic compound content was significantly greater in cultivars Basilisk and RJE when compared to the other cultivars ($F = 3.15$; $df = 3$; $P = 0.0287$) (Fig. 1A). Plants exposed to salicylic acid or nitric oxide, with or without *M. spectabilis* nymphs, exhibited a significant decrease in phenolic compound content in shoots ($F = 7.84$; $df = 5$; $P = 0.0000$); however, an isolated effect of herbivory (nymphs without inducers) was not observed, and the concentration of phenolic compounds in plants exposed to nymphs was not altered when compared to the control (Fig. 1B).

With regard to changes in the concentration of phenolics, significant interaction between the forages and inducers was found in *Brachiaria* grass roots, the feeding site of *M. spectabilis* nymphs ($F = 1.90$; $df = 15$; $P = 0.0329$) (Fig. 1C). The phenolic content was reduced in cultivars Marandu ($F = 7.23$; $df = 5$; $P = 0.0000$), Ruziziensis ($F = 4.32$; $df = 5$; $P = 0.0014$), and RJE ($F = 2.74$; $df = 5$; $P = 0.0238$) when exposed to nitric oxide, and significantly higher concentrations were recorded for Basilisk

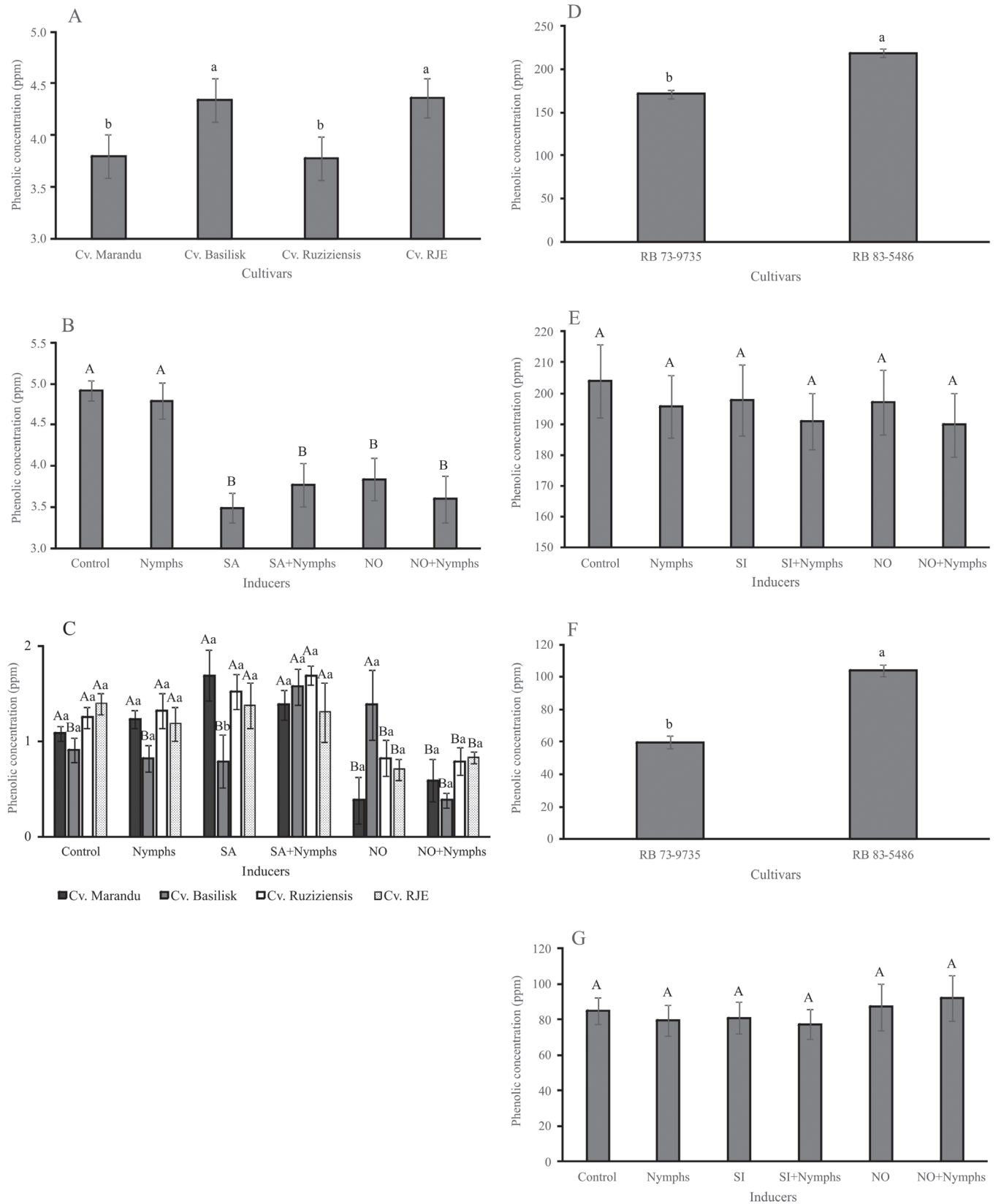


Fig. 1. Mean (\pm SE) of total phenolic compounds in *Brachiaria* shoots in relation to cultivar (A), inducer (B), and the interaction between cultivar and inducer in roots (C), in sugarcane shoots in relation to cultivar (D), and inducer (E), and in sugarcane roots in relation to cultivar (F) and inducer (G). Bars with the same low-ercase letter comparing cultivars and bars with uppercase letters comparing inducers do not differ by the Scott Knott test ($P < 0.05$).

($F = 5.03$; $df = 5$; $P = 0.0004$) for salicylic acid + nymphs and nitric oxide without nymphs compared with other treatments. In contrast, no significant alterations in phenolic content between cultivars subjected to inducers were observed, with the exception of Basilisk, when compared to salicylic acid application ($F = 4.22$; $df = 3$; $P = 0.008$) (Fig. 1C).

Regarding sugarcane, a significant variation in phenolic content in shoots ($F = 158.37$; $df = 1$; $P = 0.0000$) and roots ($F = 40.28$; $df = 1$; $P = 0.0000$) was found between cultivars with and without inducers, with higher levels in cultivar RB 83-5486 (Fig. 1D, F). However, phenolic contents did not vary in shoots ($F = 1.219$; $df = 5$; $P = 0.3086$) or roots ($F = 0.434$; $df = 5$; $P = 0.8229$) when the plants were treated with different inducers (Fig. 1E, G), and no interaction between cultivars and inducers

was found for phenolic contents in shoots ($F = 1.046$; $df = 5$; $P = 0.3974$) or roots ($F = 1.08$; $df = 5$; $P = 0.3851$).

DRY MATTER IN *BRACHIARIA* AND SUGARCANE

The production of dry matter in the shoots of *Brachiaria* grasses was not significantly different between the forage species ($F = 0.500$; $df = 3$; $P = 0.6832$) (Fig. 2A) exposed to inducers ($F = 1.64$; $df = 5$; $P = 0.1572$) (Fig. 2B), and no interaction between forage species and inducer was found ($F = 0.423$; $df = 15$; $P = 0.9688$).

In addition, dry matter in *Brachiaria* roots was not significantly different between cultivars ($F = 2.53$; $df = 3$; $P = 0.0619$) and inducers ($F =$

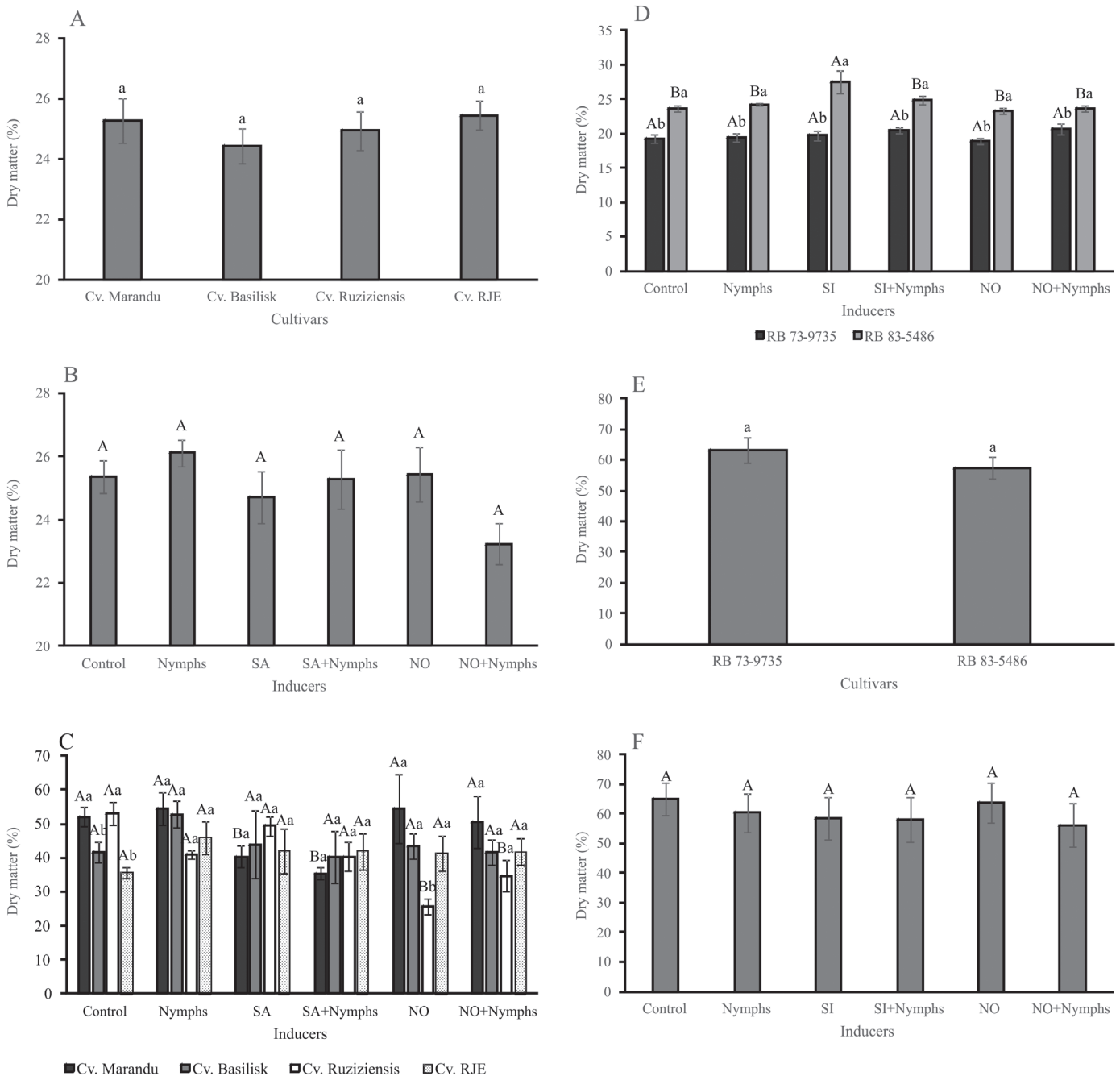


Fig. 2. Mean (\pm SE) of dry matter in *Brachiaria* shoots in relation to cultivar (A), inducer (B), and the interaction between cultivar and inducer in *Brachiaria* roots (C), in sugarcane shoots in relation to the interaction between cultivar and inducer (D), and in sugarcane roots in relation to cultivar (E) and inducer (F). Bars with the same lowercase letter comparing cultivars and bars with uppercase letters comparing inducers do not differ by the Scott Knott test ($P < 0.05$).

1.99; $df = 5$; $P = 0.0863$). However, interaction between cultivar and inducer occurred in dry matter percentages ($F = 2.18$; $df = 15$; $P = 0.0126$) (Fig. 2C). Although the root dry matter of cultivars Basilisk ($F = 0.95$; $df = 5$; $P = 0.4744$) and RJE ($F = 0.49$; $df = 5$; $P = 0.7867$) did not change due to inducers, it decreased ($F = 2.78$; $df = 5$; $P = 0.0223$) in the roots of cultivar Marandu when salicylic acid was applied to spittlebug-uninfested and -infested plants when compared to other inducers. Similar results occurred in cultivar Ruziensiensis, whereby dry matter was lower ($F = 4.36$; $df = 5$; $P = 0.0013$) in plants treated with nitric oxide with and without nymphs (Fig. 2C).

In addition to the fact that the shoots of cultivar RB 83-5486 contained a higher phenolic content, the percentage of its dry matter was greater when compared to RB73-9735, with and without inducers (Fig. 2D). Inducers failed to trigger significant alterations ($F = 1.20$; $df = 5$; $P = 0.3164$) in the dry matter of cultivar RB 73-9735, regardless of the inducers employed. However, a higher percentage of dry matter ($F = 5.95$; $df = 5$; $P = 0.001$) was recorded for cultivar RB 83-5486 when subjected to silicic acid application without *M. spectabilis* nymphs (Fig. 2D). Dry matter in sugarcane roots did not vary due to cultivar ($F = 3.14$; $df = 1$; $P = 0.0804$) (Fig. 2E), inducer ($F = 0.778$; $df = 5$; $P = 0.5688$) (Fig. 2F) or their interaction ($F = 0.786$; $df = 5$; $P = 0.5630$).

Discussion

Phenolic compounds are the most abundant and relevant groups in a plant's defense arsenal (Sharma et al. 2009; War et al. 2011). In our study, the phenolic content in the shoots and roots of *Brachiaria* cultivars and in the roots of sugarcane cultivars was not significantly different in the absence of inducers. This result suggests that there is no significant variation in the phenolic content of forages, even though they express a different degree of resistance to spittlebugs. However, the phenolic content in the shoots of *Brachiaria* grasses was altered based on cultivar when comparing all treatments. Leite et al. (2013) reported no significant variation in phenolic contents in the shoots of elephant grass genotypes in the absence of inducers (control). In addition, Silva et al. (2005) found a difference in phenolic content in the leaves of different sugarcane genotypes without inducers, with a greater concentration in a resistant cultivar. According to these authors, a high concentration of phenolic compounds may not influence the preference of the spittlebug because these cultivars are not frequently infested in the field. Similarly, Guimarães et al. (2008) reported a greater concentration of phenolic compounds in a resistant sugarcane cultivar, indicating an antibiosis resistance mechanism. Although inducers did not significantly alter the phenolic content of sugarcane, a higher concentration was found in the control treatment than in the resistant cultivar. Because *B. brizantha* is resistant to antibiosis, this change may be related to another characteristic of the plant. In fact, in the current research the phenolic content was similar in the resistant and susceptible *Brachiaria* standards.

The phenolic contents of *Brachiaria* and sugarcane cultivars attacked by nymphs did not change when compared to the control and did not indicate an isolated effect of herbivory. In their research on *Mahanarva fimbriolata* (Stål) (Hemiptera: Cercopidae) in sugarcane, Silva et al. (2005) reported a higher phenolic content in infested plant leaves, though this increase was significant only at the highest insect density. According to Lattanzio et al. (2006), replacement of the plants' defensive resources is highly relevant due to resource assets consumed during biosynthesis or in the ecological consequence of their accumulation. One approach of reducing costs is for the plant to synthesize

defense compounds only after initial damage by herbivores. It may be surmised that the nymph density in the current research was lower than that required to cause physiological alteration and induction of phenolic compounds and that the initial attack should be significant, such that production and replacement of defense compounds is measurable.

Cultivars Basilisk and RJE, which are susceptible to *M. spectabilis* according to the *Brachiaria* grass improvement program of Embrapa Gado de Leite, revealed a significantly higher phenolic content in shoots when exposed to salicylic acid or nitric oxide with or without *M. spectabilis* nymphs. However, this finding is in contrast to data in the literature, which underscores the greater amounts of phenolic content occur in plant resistant (War et al. 2015).

The concentrations of total phenolics in the roots of *Brachiaria* were significantly altered by the interaction between forage species and inducer, likely because roots are the feeding sites of *M. spectabilis* nymphs. The greatest concentration of phenolic compounds in the cultivar Basilisk, when subjected to salicylic acid + nymphs and to nitric oxide application, is favorable because this cultivar is susceptible, and physiological alterations may be indicative of decreased insect attack. However, the fact that nitric oxide, with or without *M. spectabilis* nymphs, reduced the total phenolic content in the cultivar Marandu is surprising because it is resistant to this insect; therefore, this combination is not beneficial. The above results disagree with those by Guimarães et al. (2008), who reported the greatest amount of phenolics in the roots of a resistant sugarcane cultivar. In addition, Nutt et al. (2004) states that phenolic compounds with decreasing concentrations serve as precursors of those with increasing concentrations, a fact that emphasizes the importance of phenolic content qualification and quantification when assessing induction of resistance to insect pests.

There were no significant changes in the production of dry matter in the shoots of *Brachiaria* subjected to inducers. According to Simaei et al. (2011), salicylic acid and nitric oxide regulate plant growth, and their combined effect increases leaf area, and fresh and dry weights when compared to sole applications. The concomitant application of inducers may result in greater amounts of dry matter than reported in the current study. In addition to greater amounts of dry matter in the resistant sugarcane cultivar, alterations also were invoked by the sole application of salicylic acid. However, root dry matter in *Brachiaria* and sugarcane was not altered between cultivar and between inducer.

Taken together, salicylic acid, silicic acid, and nitric oxide, as well as attack by *M. spectabilis*, triggered changes in the phenolic content in *Brachiaria* and sugarcane cultivars. Furthermore, the highest and lowest concentrations of phenolic compounds in response to the insect pest occurred in the susceptible (cv. Basilisk) and resistant (cv. Marandu) standard cultivars, respectively. Our results showed that the tested inducers failed to affect the phenolic-based defense systems of *Brachiaria* grass cultivars, and that total phenolic compound determination is not the most adequate measure for assessing resistance in these cultivars. In sugarcane, inducers did not appear to act on defense mechanisms, even though the resistant cultivar showed a greater phenolic content. Hence, the use of these chemical inducers did not trigger resistance in *Brachiaria* grasses and sugarcane.

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