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Use of extracts of *Tithonia diversifolia* and *Gymnanthemum amygdalinum* in the control of *Meloidogyne incognita*

Utilização de extratos de Tithonia diversifolia e Gymnanthemum amygdalinum no controle de Meloidogyne incognita

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ABSTRACT: *Meloidogyne incognita* (kofoid & white) chitwood. is one of the species of phytoparasitic nematodes that causes the most losses to world agriculture. The aim of the study was to evaluate the effect of the aqueous extracts of *Tithonia diversifolia* and *Gymnanthemum amygdalinum* on the hatching of juveniles of *M. incognita* and their effect on tomato plants. The extracts were obtained by dynamic infusion. For the in vitro test, microtubes containing extract and the suspension of nematode eggs were prepared. After 15 days of incubation, the number of eggs and mobile and immobile juveniles were evaluated to calculate hatch inhibition. The results demonstrated that all the tested extracts had an inhibitory effect on the hatching of *M. incognita* when compared to the conventional nematicide. In the in vivo test, tomato plants of the cultivar Santa Cruz Kada, aged three weeks, were inoculated with a suspension with 2000 eggs of *M. incognita* and subjected to application of the extracts. After 60 days, the reproduction factor (FR) and the number of galls/grams of root (NGGR) were evaluated, as well as the weight of the fresh root material (PMFR) and the aerial part of the plants (PFPA). The best results were observed in plants treated with the extract of the leaves and dry stem of *T. diversifolia*.

Keywords: Alternative control. Galls. Nematodes. Natural extracts.

RESUMO: *Meloidogyne incognita* (kofoid & white) chitwood. é uma das espécies de nematoides fitoparasitas que mais causa perdas a agricultura mundial. O objetivo do estudo foi avaliar o efeito dos extratos aquosos de *Tithonia diversifolia* e *Gymnanthemum amygdalinum* na eclosão de juvenis de *M. incognita* e seu efeito em plantas de tomateiro. Os extratos foram obtidos por infusão dinâmica. Para o ensaio *in vitro,* foram preparados microtubos contendo extrato e a suspensão de ovos de nematoide. Após 15 dias de incubação, foram avaliados o número de ovos e de juvenis móveis e imóveis para o cálculo da inibição da eclosão. Os resultados demonstraram que todos os extratos testados apresentaram efeito inibitório sobre a eclosão de *M. incognita* quando, comparados ao nematicida convencional. No teste *in vivo,* plantas de tomateiro da cultivar Santa Cruz Kada, com três semanas de idade, foram inoculadas com uma suspensão com 2000 ovos de *M. incognita* e submetidas a aplicações dos extratos. Após 60 dias foram avaliados o Fator de Reprodução (FR) e número de galhas/grama de raiz (NGGR) e também o peso da matéria fresca de raiz (PMFR) e da parte aérea das plantas (PFPA). Os melhores resultados foram observados nas plantas tratadas com o extrato das folhas e do caule seco de *T. diversifolia.*

Palavras-chave: Controle alternativo. Extratos naturais. Galhas.Nematoides.

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INTRODUCTION

Meloidogyne incognita (Kofoid & White) Chitwood, is considered one of the nematode species that causes most losses to world agriculture. This is due to its wide geographical distribution and its wide range of hosts.

Its life cycle comprises an egg phase from which hatches a second stage juvenile (J2) representing the infective phase. It penetrates the root with the help of its stylus and moves through the cells to the differentiation zone of the vascular cylinder. After penetration, the nematode goes through two more stages until adulthood (FERRAZ; BROWN, 2016).

Its parasitism leads to hypertrophy and hyperplasia of vascular cylinder cells, which form root swellings, which are popularly known as galls (ALMEIDA-ENGLER et al., 2016). The structural deformations caused in the root system cause symptoms that can be noticed in the aerial part of susceptible plants such as wilting, yellowing, defoliation among others.

The adult female is a sedentary endoparasite with pear-shaped body and produces around 500 eggs that may be exposed to the soil or remain inside the root protected by a gelatinous substance secreted by it (FERRAZ; BROWN, 2016).

The development and maintenance of *M. incognita* populations strongly depends on the success of embryogenesis and hatching processes (CAMPOS, 2008). Strategies aimed at regulating nematode populations in certain areas should have the ability to influence aspects related to their reproduction.

The egg stage is considered the toughest phase of the nematode's life, as its shell prevents even very small molecules from passing through. However, before hatching, the eggshell of gall nematodes undergoes transformations that make it permeable (ROBINSON, 2009). This permeability allows external factors, such as toxic substances, to interfere with embryonic development (DIAS-ARIEIRA, et al. 2008).

Substances of plant origin such as alkaloids, flavonoids, terpenes, limonoids, rocaglamides, furanocoumarin, chromos and acetylenes, commonly found in plants, can act to inhibit the hatching process by presenting nematicidal properties (CHITTWOOD, 1992; VIEIRA; MAFEZOLI; BIAVATTI, 2007).

The use of botanical extracts with nematicidal or nematostatic properties is a promising alternative in the population control of phytonematoids. Several plants have already been studied and have been shown to be effective in tests performed on populations of different nematode species (FERRIS; ZENG, 1999; SLOMP, 2009; FERREIRA et al. 2013; MOREIRA

et al. 2015; SANGI, 2018). The substances in these compounds can act through various mechanisms in the different phases of the life cycle of these plant parasites.

Tithonia diversifolia (Hemsl.) Gray, popularly known as Mexican sunflower, and *Gymnanthemum amygdalinum* Baker, known as figatil or boldo baiano are species of the Asteraceae family, originating in Mexico and Africa respectively, traditionally consumed for their antimicrobial, pharmacological and phytotherapeutic activities (GUALBERTO et al., 2011; AFONSO et al., 2015; BOEING et al., 2016). Other species of the same family have been shown to be effective in inhibiting *M. incognita* hatching (FERREIRA, et al. 2013).

In general, the most outstanding compounds in the chemical study of plants of the Asteracea family are flavonoids and terpenoids (VERDI et al., 2005). Phytochemical analysis indicates that *G. amygdalinum* contains compounds such as flavonoids and phenolic acids that are responsible for antimicrobial and enzyme inhibitory activity (CARVALHO, 2014). In addition to these, compounds such as steroids and saponins have also been found in *T. diversifolia* (TONA et al., 1998).

Thus, based on the available literature, it is possible to assume that the plants in question have the potential to conduct studies on the viability of their use as an alternative control of *M. incognita.*

In this sense, the aim of this study was to evaluate the effect of aqueous extracts of leaves, stems and inflorescences of *T. diversifolia* and *G. amygdalinum* on hatching of *M. incognita* juveniles in vitro and to test their efficiency in nematode control in tomato plants. (*Solanum lycopersicum) in vivo.*

2 MATERIAL AND METHODS

The work was carried out at the Brazilian Agricultural Research Corporation / Rondônia-EMBRAPA Agroforestry Research Center located in the city of Porto Velho - Rondônia. The in vitro test was performed and evaluated in the Phytopathology laboratory and the in vivo experiment was installed in a greenhouse.

Plant collections for extract preparation were performed after approval of the registration in Sisgen under number A7126B3. The plant materials used for the preparation of the extracts came from the Colorado do Oeste Country, Rondônia in the coordinates 13 ° 7'1.28 "S 60 ° 29'19.05" O. They were collected in late October 2018 and included leaves, stems and inflorescences of the middle third of plants approximately 110 days old, separately packed in properly identified plastic bags and transported to EMBRAPA's Phytopathology laboratory. According to the Koppen classification, the region's climate is Aw, hot and humid, with two

well-defined climatic seasons, an average temperature of 22.1 ° C and an average annual rainfall of 1906 mm (MERKEL, 2019).

For the preparation of the extracts, parts of the materials were placed in paper bags and placed in a forced ventilation oven at 65º C for a period of 72 hours to obtain the dry material and another part of the fresh material was immediately submitted to the extraction process.

Extracts from each part of the fresh and dried plants (stems, leaves and inflorescences) were prepared (Table 1). The different materials were crushed with the help of an industrial blender in order to obtain the finest material possible. Then 10 grams of each was separated and weighed and placed in Erlenmeyer flasks where 100 ml of sterile mineral water was added, which was the extractor. The vials were capped under shaking in a refrigerated Shaker incubator at 100 rpm for 24 hours at 25° C. At the end of the process, the extracts were filtered through gauze and nylon cloth, stored in hermetically sealed bottles and placed in the freezer for later use.

The inoculum used in the *in vitro* test was obtained from pure populations of *M. incognita* from the experimental field of Embrapa de Ouro Preto do Oeste, identified by Santos (2017) as *M. incognita* EI2 and kept in chicory plants *Cichorium endivia* variety. 'Valença' cultivated in pots in protected environment at EMBRAPA of Porto Velho.

Table 1. Species, Part of the plant used, preparation of extracts used in the in vitro experiment with *M. incognita*

Plant	Used part	Preparation	Code
Tithonia diversifolia	Inflorescence	Dry	TDIS
Tithonia diversifolia	Stalk	Dry	TDCS
Tithonia diversifolia	Inflorescence	Fresh	TDIF
Tithonia diversifolia	Leaf	Dry	TDFS
Tithonia diversifolia	Leaf	Fresh	TDFF
Tithonia diversifolia	Stalk	Fresh	TDCF
Gymnanthemum amygdalinum	Stalk	Dry	GACS
Gymnanthemum amygdalinum	Inflorescence	Dry	GAIS
Gymnanthemum amygdalinum	Leaf	Dry	GAFS
Gymnanthemum amygdalinum	Inflorescence	Fresh	GAIF
Gymnanthemum amygdalinum	Stalk	Fresh	GACF
Gymnanthemum amygdalinum	Leaf	Fresh	GAFF

For egg extraction, the plants were collected and the aerial part discarded. The roots were gently washed and cut into pieces of approximately 1 cm and subjected to the blender method combined with the sucrose sieving and centrifugation method proposed by Coolen and D'herd (1972).

The suspension resulting from the extraction process was deposited in a Becker and pipetted with a semi-automatic micropipette. The quantification was performed with the aid of an excavated slide, where the eggs were counted under a stereoscopic microscope. Six counts

were performed to calculate the egg average and then calibrate the suspension which resulted in a concentration of 50 eggs / 100µl of suspension.

To test the effect of *T. diversifolia* (Hemsl) Gray and *G. amygdalinum* Baker crude extracts on hatching and mortality of *M. incognita* juveniles, Eppendorf-type microtubes with a capacity of 250 µl were deposited with 100µl of extract together with 100µl of the suspension, containing 50 nematodes eggs. As controls, sterile mineral water and Carbofurano nematicide (Furadan) were used according to the manufacturer's recommended dose (20 ml / l). Each microtube represented one experimental unit. A completely randomized design with 14 treatments and 6 repetitions was used.

The Eppendorfs were placed on support racks and were packed with aluminum foil, identified and taken to the B.O.D incubator at 25°C for 15 days. After this period, mobile and immobile second stage (J2) eggs and juveniles were counted from each tube. With the data obtained, the adjustment calculations were performed due to the inhibition of hatching by water and then the calculation of the inhibition of hatching by extracts and nematicide. Results were submitted to analysis of variance (ANOVA) and means compared by Scott-Knott test at 1% probability.

The *in vitro* experiment was carried out in polypropylene pots with capacity for two liters of substrate, prepared with autoclaved soil and sand at a ratio of 1: 1 (v / v). A two-week-old tomato seedling of Santa Cruz Kada cultivar was planted in each pot. The inoculation was done one week after the definitive planting of tomato seedlings. The suspension used contained an average of 2.000 *M. incognita* eggs and it was deposited on the substrate in approximately 3 cm glass-hole holes.

Twenty ml of extract was applied per pot and eight extracts with three controls were tested (TABLE 2). The first application of the extracts occurred 24 hours after inoculation and the second 30 days after the first application. The application of the nematicide occurred only once. The evaluation of the experiment took place 60 days after inoculation. For that, the plants were removed from the pots and had the aerial part and the roots separated. The roots were washed and shaded to remove excess moisture.

Table 2. Identification of plant species, part used and preparation of extracts used in the in vivo experiment with *M. incognita*

Code	Species	Used part	Form of extraction
GAFF	*Gymnanthemum amygdalinum*	Stalk	Fresh
GAFS	*Gymnanthemum amygdalinum*	Leaf	Dry
GSCS	*Gymnanthemum amygdalinum*	Stalk	Dry
GACF	*Gymnanthemum amygdalinum*	Stalk	Fresh
TDFF	Tithonia diversifolia	Leaf	Fresh
TDFS	Tithonia diversifolia	Leaf	Dry
TDCF	Tithonia diversifolia	Stalk	Fresh

TDCS	Tithonia diversifolia	Stalk	Dry
NEMT	Furadan®	-	-
ÁGUA	Mineral water	-	-
NIN	Non inoculated	-	-

The variables analyzed were Fresh Airway Weight (PFPA), Fresh Airway Weight (PFR), Dry Airway Weight (PSPA) and Dry Root Weight (PSR). In addition, 3 grams of roots were separated where the number of galls was counted and extracted for egg counting and calculation of the Reproduction Factor. The experimental design was completely randomized and had 8 treatments and three witnesses with six replications. Results were submitted to analysis of variance (ANOVA) and means compared by Scott-Knott test at 5% probability.

3 RESULT AND DISCUSSION

In the in vitro experiment 12 raw aqueous extracts from different preparation forms and parts of the two plants were tested. According to the analysis of variance (ANOVA) there were significant differences in the observed results (Table 3). The witnesses Water and Nematicide showed significant differences, which denotes the reliability of the test.

Table 3. Summary of variance analysis of the effects of aqueous extracts of *T. diversifolia* and *G. amygdalinum* on inhibition of *M. Incognita* compared to water and commercial nematicide control performance

FV	GL	SQ	QM	F
Treatments	13	28566.69	2197.44	148.261**
Extracts (Ext)	11	188.20	17.11	1.1543^{NS}
Witnesses (Wit)	1	7901.23	7901.23	533.09**
Ext x Test	1	20477.27	20477.27	1381.6**
Waste	70	1037.50	14.82	-
Total	83	29604.19	-	-
$AVERAGE_{overall}$	89.34	-	-	-
$Average_{extracts}$	95.71	-	-	-
$Average_{witnesses}$	51.09	-	-	-
CV(%)	4.31	-	-	-

FV: Source of variation, GL: degrees of freedom, SQ: Sum of squares, QM: Mean square, F: F statistic which is hypothesized that there is no difference in mean treatments, **: significant at 1% probability. NS: not significant.

Regarding the extracts, they did not show significant differences among themselves, however they contrasted with the witnesses, demonstrating that all extracts used in the experiment were efficient in the control of second stage juveniles of *M. incognita.* The differentiated positive response of some extracts to chemical control indicates that they may be as or more efficient than conventional nematicide.

The average hatching inhibition caused by the use of the extracts was higher than the average of the experiment, which also indicates the possibility of finding more efficient extracts than the commercial product (Figure 1).

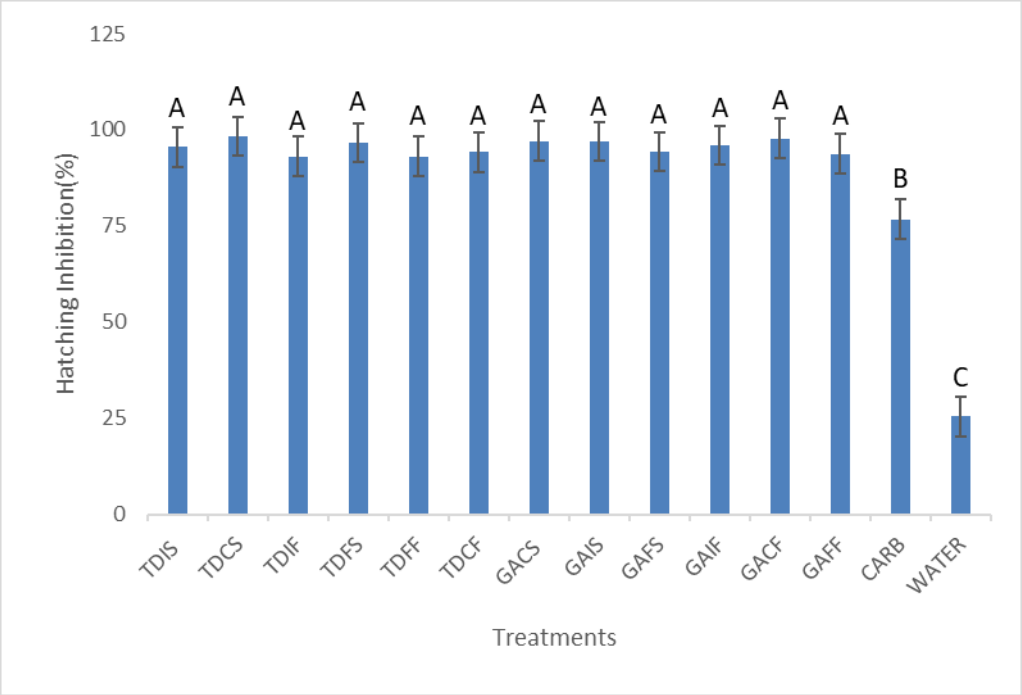

Figure 1. Effect of aqueous extracts of *T. diversifolia* and *G. amygdalinum* on hatching inhibition of second stage juveniles of *M. incognita in vitro.*

Both *T. diversifolia* and *G. amygdalinum* have been shown to have phytochemicals that can interfere with the embryonic development process of *M. incognita.* There were no differences in effect between the parts used, it is assumed that plants produce the compounds in a well-distributed manner by leaves, stems and inflorescences and the concentration in these parts is high enough that prevented determining which is the most efficient.

Lezcano et al. (2012), performed the phytochemical characterization of the edible fraction of *T. diversifolia* in different stages of its physiological cycle and in the rainy and dry period where they detected the presence of primary and secondary amines, free phenols, tannins, triterpenes, steroids that presented with light form, and the alkaloids presented the highest amounts. However, no variations were observed in the quantitative evaluations of the compounds between leaves and stems.

No studies were found that reported the variation in metabolite production in the different parts of *G. amygdalinum*. However, Afonso et al. (2015) found significant seasonal effects on relative abundances of metabolites, such as alkaloids, present in *G. amygdalinum* leaves.

Regarding the preparation method, the results observed in the treatments with aqueous extracts obtained from the dried plant material did not differ from the results from the extracts

made with fresh plant material. This observation tells us that bioactive compounds responsible for the inhibition of juvenile hatching do not get lost during the drying process and therefore are not volatile.

The analysis of variance showed that all parameters observed in the test performed to verify the effect of crude aqueous extracts of *T. diversifolia* and *G. amygdalinum* on tomato plants in vivo showed significant differences between themselves and the controls.

Regarding the number of galls per gram of root, the extracts prepared with fresh and dry *T. diversifolia* stem (TDCF and TDCS) differed from the other extracts and the water control, showing the number of galls similar to the synthetic nematicide treatment (Figure 2). The other extracts had no effect on gall reduction when compared to water control.

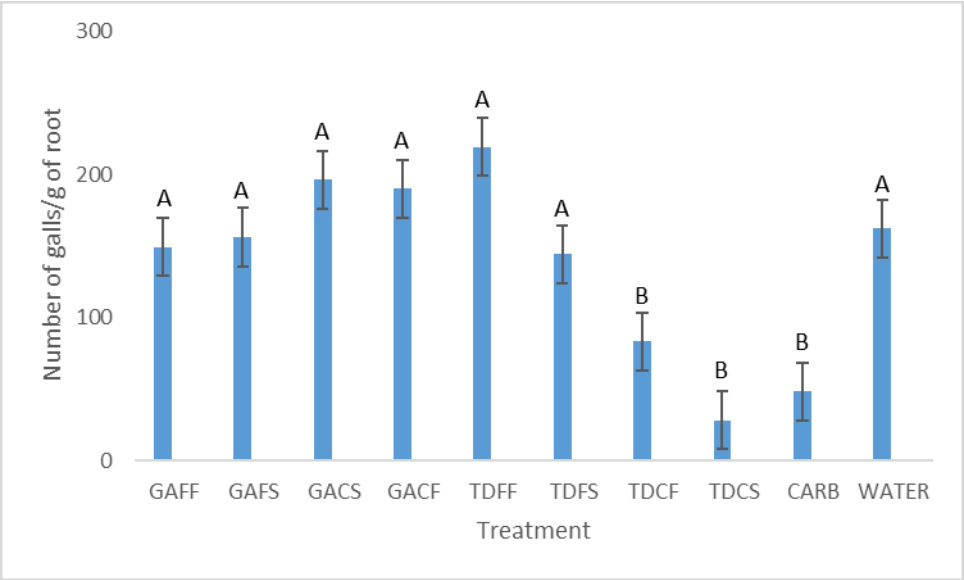

Figure 2. Effect of aqueous extracts of *T. diversifolia* and *G. amygdalinum* on the number of galls (NG) of *M. incognita* in tomato roots under greenhouse conditions.

The TDCS extract, when added to the soil, decreased the number of galls per gram of root of treated plants by 82.61% and TDCF by 48.76% in relation to water control. Gardiano et al. (2009), working with aqueous extracts of mint, burdock and castor oil observed a reduction of 75.60%, 65.73% and 54.40%, respectively.

Mateus et al. (2014) found reductions in the number of galls in tomato roots of 64.5% and 71.0% when evaluating the effect of Gervão (*Stachytarpheta cayennensis LC. Rich. Vahl*) and stinging (*Bidens pilosa* L.).

The reproduction factor (RF) represents the relationship between the final population of the nematode and the initial population (2.000 eggs) of each treatment. As observed (Figure 3), extracts obtained from fresh stem and dried leaves of *T. diversifolia* (TDCF and TDFS) were

the treatments that presented the lowest RF values as efficient as nematicide for reproduction control from *M. incognita.*

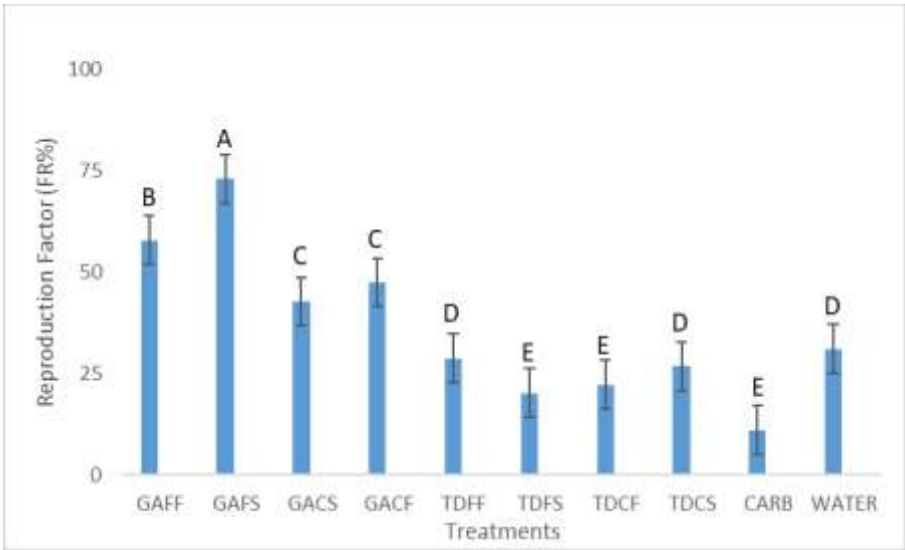

Figure 3. Reproduction factor (RF) of *M. incognita* in tomato roots submitted to treatments with aqueous extracts of *T. diversifolia* and *G. amygdalinum.*

Considering water-only treatment as a standard, it is possible to observe a reduction of 34.79% in FR when tomatoes were treated with TDFS and 28.28% when treated with TDCF. The treatments VCFS, VCFF, VCCF and VCCS differed from the two controls, and they presented high RF values. The TDFF and TDCS treatments, although presenting a small reduction in relation to the water treatment, did not differ significantly, which is supposed to have no effect on *M. incognita* reproduction in tomato plants. In this case only two extracts, made with *T. diversifolia* leaves and stems, had an effect on nematode reproduction.

Extracts belonging to the same plants showed different behavior in the experiment. This can be justified because according to Irulandi (2008) the solar incidence and the temperature to which botanical extracts are subjected can lead to photodegradation of the active compounds responsible for controlling the pathogen.

Regarding fresh shoot weight (PFPA), the controls varied significantly, which shows that these treatments influence the increase of fresh matter and the shoot development. *T. diversifolia* dry stem (TDCS) and *G. amygdalinum* fresh leaf (VCFF) and dry leaf (VCFS) extracts showed the best results. Plants treated with such extracts had better performance than those submitted to treatment with nematicide.

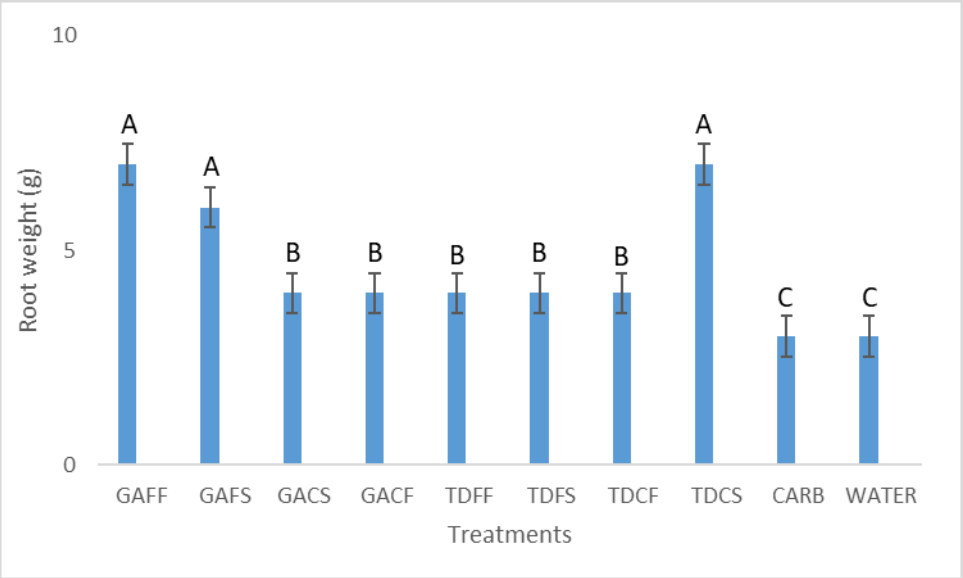

Figure 4. Weight of fresh root matter shoots of tomato plants inoculated with *M. incognita* submitted to treatments with aqueous extracts of *T. diversifolia* and *G. amygdalinum.*

All the extracts used provided a greater weight of fresh root matter (PFR) to the treated plants when compared to those treated with nematicide and water (Figure 4).

Considering the PFPA and PFR parameters, it is noted that the extracts did not interfere with the growth of the plants and in some cases they may have increased the growth.

Likewise, Ferreira et al. (2013) observed an increase in the weight of the aerial part of plants treated with aqueous extracts of vedelia (*Sphagneticola trilobata*), Mexican sunflower (*Tithonia diversifolia*), marigold (*Tagetes erecta*) and other plants of the Asteraceae family, which suggests that plants of such a family have properties that stimulate the growth of plants. The use of natural extracts rich in macro and micronutrients can promote better performance in plant growth.

The results obtained by Gardiano et al. (2008), when they made use of aqueous extracts of guinea (*Petiveria alliacea* L.), fennel (*Foeniculum vulgare* Mill) and sunflower (*Helianthus annus* L.) by means of foliar spraying of tomato plants, demonstrate a situation similar to the current study. Such extracts reduced the number of galls by 61%, 40.4% and 35.6%, respectively and provided an increase in the weight of the fresh weight of the root and aerial part of the tomato plants, however there was no significant difference in the reduction of the number of eggs in relation to the control.

4 FINAL CONSIDERATIONS

The results show that, under the conditions in which the in vitro experiments were performed, all extracts of *T. diversifolia* and *G. amygdalinum* were efficient in inhibiting the

outbreak of *M. incognita* juveniles. In the field, the extracts that presented the best results in reducing the number of galls and reproduction factor (FR) of *M. incognita* in tomato plants were those obtained from *T. diversifolia* stem and dried leaves.

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