*Research Journal of Chemistry and Environment_____*Vol. **24** (12) December **(2020)**

Res. J. Chem. Environ.

One-pot synthesis and insecticidal activity of 5-amino-1-aryl-1*H*-pyrazole-4-carbonitriles

Plem Silvana Cristina[1,2], Machuca Laura Marcela[2,3], Gutierrez Carolina Guadalupe[1,2], Coll Aráoz MaríaVictoria[2,4],
Van Nieuwenhove Guido Alejandro[2,5], Virla Eduardo Gabriel[2,5] and Murguía Marcelo César[1,2]*

1. Laboratorio de Medio Ambiente. INTEC (UNL-CONICET Santa Fe). Güemes 3450. (3000) Santa Fe, ARGENTINA
2. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290 (C1425FQB) Buenos Aires, ARGENTINA
3. Instituto de Investigaciones Científicas (IDIC), Facultad de Ingeniería y Tecnología, Universidad de la Cuenca del Plata (UCP). Corrientes 57, (3450) Goya, Corrientes, ARGENTINA
4. División Control Biológico. PROIMI-Biotecnología (CONICET Tucumán). Avda. Belgrano y Pasaje Caseros. (4001) San Miguel de Tucumán, Tucumán, ARGENTINA
5. Fundación Miguel Lillo, Instituto de Entomología, Miguel Lillo 251.(4001) San Miguel de Tucumán, Tucumán, ARGENTINA
*mmurguia@santafe-conicet.gov.ar

Abstract
Synthesis of 5-amino-1-aryl-1H-pyrazole-4-carbonitriles was performed by direct condensation of (ethoxymethylene)malononitrile and aryl hydrazines. The resulting N,N-containing heterocycles possess insecticidal properties relative to microlepidoptera species, plant phatogens. The insecticidal activity of four novel synthetic N-aryl pyrazoles to Tuta absoluta larvae was assessed. Fipronil, a well-known aryl pyrazole insecticide, was also tested as the positive control. A generalized linear model reported significant differences in efficacy and tomato leaf consumption among the different treatments.

Fipronil treatment was the most effective one (100% mortality after 48 h), followed by 5-amino-1-phenyl-1H-pyrazole-4-carbonitrile treatment (3c) (75% mortality after 48 h) and 5-amino-1-(2,6-dichloro-4-(trifluoromethyl)-phenyl-1H-pyrazole-4-carbonitrile treatment (3b) (60% mortality after 48 h). These compounds, with simpler structures than Fipronil could be used as novel insecticides.

Keywords: N-aryl pyrazoles, Chemical pest control, *Tuta absoluta*, Lepidoptera, *Solanum lycopersicum* L.

Introduction
One of the main concerns in crop protection is the development of resistant pests. The continued application of traditional pesticides has often led to the development of resistance, thus bringing about enormous losses in crop production. In the search for novel insecticides, many compounds containing a pyrazole nucleus have been developed as herbicides, insecticides and fungicides. Pyrazoles are heterocyclic compounds that can act as binucleophiles with a broad spectrum of biological activities[1].

Fiproles, a specific serie of aryl pyrazoles, were developed as a compound class in 1985 as a spin-off of herbicidal research that can act as neurotoxins, antagonists on γ-aminobutyric acid (GABA)-gated chloride channels[2]. One of the references is Fipronil [5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl) pyrazole-3-carbonitrile] which blocks GABA receptors in insects, disrupting nervous system function and causing death by hyperexcitation. Fipronil is highly effective against a significant range of insect pests and has become a cornerstone in control programs for both crop and noncrop insect pests worldwide[3].

Fipronil is currently registered in many countries for insect pests control for different crops, ranging from row crops such as rice, corn, potatoes and small grains, to ornamentals, mangoes and chili peppers. Fipronil exhibits <500-fold selective toxicity to insects over mammals, primarily because of the affinity differences in receptor binding between insect and mammalian receptors[2].

On the other hand, the tomato leafminer, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), a neotropical oligophagous pest of solanaceous crops, is a major pest of tomato crops worldwide[4]. This pest causes severe crop losses and has been listed under EPPO A2 (European Plant Protection Organization) alert list of harmful insects for which quarantine measures are recommended if they are accidentally introduced[5]. It is native to South America[5] with the exception of the Andean Region at altitudes higher than 1.000 m.

The preferred host is tomato (*Solanum lycopersicum* L.), where female adults lay eggs on the above-ground portions of the plant (leaves, shoots, flowers) including the fruit[6] but it can also lay eggs and feed on other solanacean species, such as *S. tuberosum* L. (potato), *S. melongena* L. (egg plant), *S. muricatum* Aiton (sweet pepper, cucumber), *Nicotiana tabacum* L. (tobacco), and other non solanacean species such as *Phaseolus vulgaris* L. (bean), *Physalis peruviana* L. (cape gooseberry) and *Capsicum annuum* L.(pepper)[5,7].

After the initial detection in eastern Spain in 2006, it rapidly invaded various other European countries and spread throughout the Mediterranean basin[7]. In 2012, this pest was detected in Africa and Asia[8]. It has been demonstrated that if no control measures are taken, this pest can cause up to 80% yield losses in tomato crops. Moreover, it can lead to secondary infestations[9].

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Larvae of *T. absoluta* usually attack the leaves by eating the mesophyll and leaving the epidermis undamaged. They also destroy the developing fruit by mining its flesh. The aggressiveness of this pest is towards tomato and its high biotic potential. In addition to its ability to easily develop resistance to the most commonly used insecticides, it can cause severe constraints on the production, commercialization and export of this economically important fruit[7].

The current control strategies for *T. absoluta* include pheromone traps and insecticides application[9]. In South America, *T. absoluta* control is based mostly on synthetic pesticides, and more than 16 types of neurotoxic insecticides are applied on a weekly basis up to 14 times per growing season[10]. Initially, the only insecticides used against the tomato leaf miners were organophosphates, which were gradually replaced by pyrethroids during the 1970's. In the early 1980's, cartap and thiocyclam were introduced showing excellent efficacy at that moment. During the 1990's, insecticides with novel modes of action e.g. abamectin, spinosad, insect growth regulators such as acylurea or tebufenozide, and chlorfenapyr were introduced[11].

In general, chemical control against *T. absoluta* has been inefficient due to the concealed (within-leaf) feeding behavior of these larvae. Thus, tomato producers usually have to increase phytosanitary use as the season progresses, which may lead to an increase of around 70% of the total pest management costs[9]. A high level of resistance to some of these compounds has been reported[11]. For these reasons, more rational and economical control alternatives are needed for the sustainability of the system.

Fipronil has been used against a broad spectrum of pests and in a variety of formulations. However, it is a highly toxic compound to many beneficial insects, mammals (including human beings), fish and birds as well as its degradation products[2]. It has also been reported that Fipronil and its residues have the effect of persistence and are also toxic to beneficial organisms through contact action[2].

Considering the impact of this compound in the ecosistem and non-target species of terrestrial, freshwater and marine environments[3], the purpose of this work is to develop other less toxic N-aryl pyrazole insecticides with fewer biosynthetic steps which might result in more economical and ecofriendly alternatives. For this study, four N-aryl pyrazoles synthesized in our laboratory were assayed against *T. absoluta* larvae.

Moreover, acute toxicity on *Daphnia magna* was also tested for the most active compounds. *D. magna* was used as the bioindicator of toxicity because it is versatile, sensitive and commonly used as a reference culture collection for toxicity assays in the environment[12]. This cladoceran not only used to evaluate the toxicity of chemicals in aquatic systems but

also plays an important role in building up regulatory criteria by environmental agencies (e.g. Environmental Protection Agency, Organization for Economic Cooperation and Development)[13].

Material and Methods

General procedure: All the aryl pyrazoles were characterized by ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, COSY, HSQC, HMBC and mass spectrometry (MS). Yields were quantified by GC (internal standard method using a witness of each pyrazole) on a DANI Master GC chromatograph equipped with 5 % diphenyl, 95 % dimethylpolysiloxane capillary column (30 m × 0.53 mm, 0.5 μm film thickness) and a flame ionization detector (FID). Column chromatography was performed on silica gel (70 - 230 mesh ASTM), high-purity grade, pore size 60 Å.
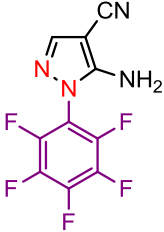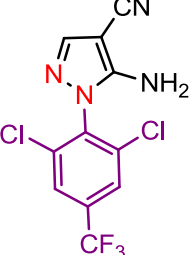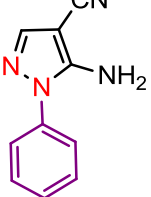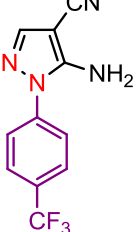
Ultra performance liquid chromatography and mass-spectrometry (UPLC-MS) were performed on an H-CLASS equipped with a SQD2 detector (Waters). ^{1}H NMR (300 MHz), ^{13}C NMR (75 MHz). ^{19}F NMR (300 MHz), correlation spectroscopy (COSY), heteronuclear single-quantum correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) were recorded at 20 °C on a Bruker Avance 300 MHz spectrometer in CDCl$_3$ using TMS as internal standard. Melting points were recorded on a Büchi b-540 micro melting point apparatus and were uncorrected.

One-pot synthesis of 5-amino-1aryl-1*H*-pyrazoles-4-carbonitriles (3a-d): Commercial aryl hydrazines (1a-d) (1.2 mmol) in ethanol absolute (2 mL) were added in a glass reactor of 25 mL under controlled atmosphere (N$_2$) and magnetic stirring. Afterwards, 1.2 mmol of (ethoxymethylene)malononitrile (2) was added slowly and the solution was carefully brought to reflux for 4 h and the crude was cooled at room temperature[14]. Then, the mixture was diluted with ethyl acetate (50 mL) and washed with water (30 mL). The organic phase obtained was dried over Na$_2$SO$_4$, filtered, and the organic solvent was evaporated under vacuum.

The products of the reaction were purified by column chromatography on silica gel with an hexane:ethyl acetate gradient mixture (8:1 to 3:1) as eluent.This general procedure resulted in the purification of compounds 3a-d (63, 47, 84 and 66% yields respectively).

The products obtained (Figure 1), once purified, were characterized by different types of spectroscopic analysis such as nuclear magnetic resonance (RMN), ultra resolution liquid chromatography (UPLC) and gas cromatography (CG). For the special case of aryl hydrazine hydrochloride 1b, a previous step of neutralization with Et$_3$N (1.0 mmol) in ethanol (2 mL) at 0 °C was needed[15]. Then, the reaction procedeed according to the methodology previously described. Table 1 described the analytical data and characterization of the synthesized compounds 3a-d.

Table 1
**Analytical and spectral data for synthesized compounds 3a-d**

Compound	Yield (%)	Mp (°C)	Selected Spectral Data			
			^{1}H-NMR δ (ppm)	^{13}C-NMR δ (ppm)	^{19}F-NMR and 2D-NMR δ (ppm)	HRMS
5-Amine-1-(perfluorophenyl)-1*H*-pyrazole-4-carbonitrile (**3a**)[a]	63	135.4-136.4	4.69 (s br, 2H, NH₂); 7.73 (s, 1H, CH)	76.4(C); 113.1 (C); 143.4 (CH); 152.1 (C). It was not possible to assign the other carbons corresponding to this molecule	−158.95 (t, 2F); −148.58 (t, 1F); −143.24 (d, 2F) COSY (δH/δH): 7.76/7.76, 4.72/4.72. HSQC (δH/δC): 7.75/143.4 HMBC (δH/δC): 7.76/76.4,7.76/112.1, 7.76/152.1	Calcd. for C₁₀H₃F₅N₄⁻: 273.0194 Found: 273.0206
5-Amino-1-(2,6-dichloro-4-trifluoromethyl)phenyl-1*H*-pyrazole-4-carbonitrile (**3b**)[b]	47	163.5-164.8	4.70 (s br, 2H, NH₂); 7.72 (s, 1H, CH); 7.77 (s, 2H, CH)	75.4 (C); 113.4 (C); (116.5, 120.1, 123.7, 127.3, q, ^{1}J = 273.58Hz -CF₃); 126.2 (CH); (133.8, 134.2, 134.7, 134.9,q, ^{2}J = 34.59Hz-CF₃);136.7(C); 142.9 (CH); 151.4(C)	−63.22 (s, 2F) COSY (δH/δH): 7.77/7.77, 7.72/7.72, 4.69/4.69 HSQC (δH/δC): 7.77/126.2, 7.72/142.9 HMBC (δH/δC): 7.77/75.4, 7.77/134.7, 7.77/136.7	Calcd. for C₁₁H₅Cl₂F₃N₄Na⁺: 342.9736 Found: 342.9731
5-Amino-1-phenyl-1*H*-pyrazole-4-carbonitrile (**3c**)[c]	93	138.5-139.6 (140.0-140.5)[15]	4.81 (s br, 2H, NH₂); 7.40 - 7.55 (m, 5H, CH); 7.57 (s, 1H, CH)	75.6 (C); 114.1 (C); 124.1 (CH); 128.8 (CH); 129.8 (CH); 136.9 (C); 141.2 (CH); 150.0 (C)	Ref. 14	Ref. 14
5-Amine-1-(4-fluorophenyl)-1*H*-pyrazole-4-carbonitrile (**3d**)[d]	47	178.5-179.8 (177.0-178.0)[14]	4.61 (s br, 2H, NH₂); 7.19 - 7.26 (m, 2H, CH); 7.47 - 7.51 (m, 2H, CH); 7.63 (s, 1H, CH)	76.1(C); 113.8 (C); 116.8 - 117.1 (CH, 1J = 23.09 Hz); 126.4 - 126.5 (CH, 2J = 9.50 Hz); 132.9 (C); 141.3 (CH); 149.8 (C); 160.7 and 164,0 (C, 2J = 250.34 Hz)	−110.96 (s, 1F)	Ref. 14

a. White solid product. ^{1}H NMR [300 MHz (CDCl₃)]; ^{13}C NMR [75 MHz]; ^{19}F NMR [300 MHz]; ^{1}H-^{1}H COSY NMR [300 MHz, CDCl₃] (δH/δH); ^{1}H-^{13}C HSQC NMR [300 MHz, CDCl₃] (δH/δC); ^{1}H-^{13}C HMBC NMR [300 MHz, CDCl₃] (δH/δC); HRMS (ESI−)
b. White solid product. ^{1}H NMR [300 MHz (CDCl₃)]; ^{13}C NMR [75 MHz]; ^{19}F NMR [300 MHz]; ^{1}H-^{1}H COSY NMR [300 MHz, CDCl₃] (δH/δH); ^{1}H-^{13}C HSQC NMR [300 MHz, CDCl₃] (δH/δC); ^{1}H-^{13}C HMBC NMR [300 MHz, CDCl₃] (δH/δC); HRMS (ESI+)
c. White solid product. ^{1}H NMR [300 MHz (CDCl₃)]; ^{13}C NMR [75 MHz]
d. White solid product. ^{1}H NMR [300 MHz (CDCl₃)]; ^{13}C NMR [75 MHz]; ^{19}F NMR [300 MHz]
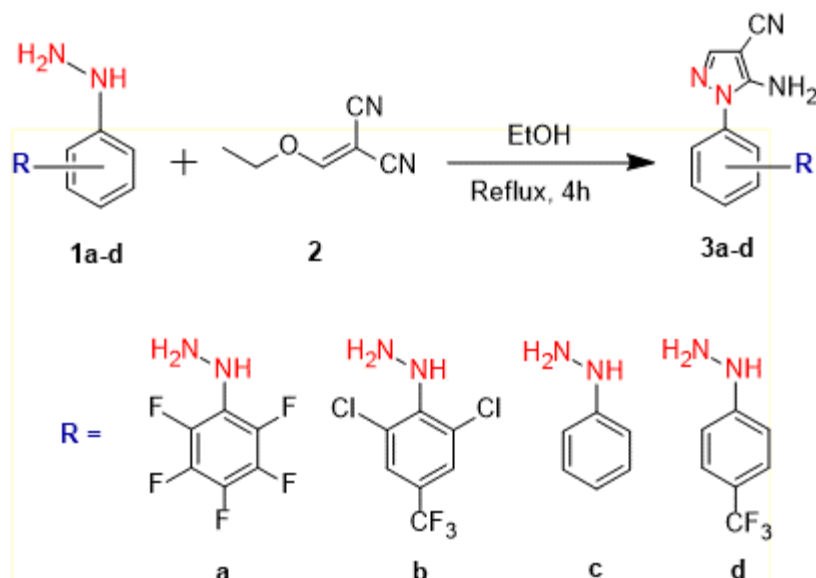
Figure 1: One-pot synthesis of 5-amino-1-aryl-1*H*-pyrazole-4-carbonitrile (3a-d) from aryl hydrazines (1a-d) and (ethoxymethylene)malononitrile (2). Aryl hydrazines (1a-d): 1a: (perfluorophenyl)hydrazine; 1b: 2,6-dichloro-4-(trifluoromethyl)phenylhydrazine; 1c: phenylhydrazine; 1d: 4-(trifluoromethyl)phenylhydrazine

Bioassays
Origin of larvae and laboratory rearing: *T. absoluta* colony was established with individuals (larvae) collected in Lules, Tucumán, Argentina (26º 56'S - 65°18'W). Larvae were placed in aluminum breeding cages (50 x 50 x 50 cm) with the lateral and upper sides covered with nylon mesh (organdy type) to promote aireation. Potted *S. lycopersicum* var. Regina plants were used to feed and maintain the colonies which were reared in a greenhouse in San Miguel de Tucumán (S 26°48' - W 65°14') under the following conditions: 20 to 30°C, 45-90% RH, and natural photoperiod.

Preparation of N-aryl pyrazole solutions; Four solutions at 1000 ppm of each N-aryl pyrazoles (3a-d) were prepared and a solution of Fipronil at 1000 ppm was prepared by disolving the compounds in a mix of distilled water:acetone (80:20) and 0.01 % Tween® 80 (T1-T4, and Fipronil T5). Also, a distilled water:acetone (80:20) and 0.01 % Tween® 80 solution were prepared as control (T6). 2cm diameter disks were cutout from the tomato leaves (*S. lycopersicum* var. Regina). Treatments (T) consisted of soaking a disk in a solution of: 3a (T1), 3b (T2), 3c (T3), 3d (T4), and Fipronil (T5) for 2 s (Figure 2); afterwards the disks were left until solvent evaporation at room temperature.

Application of treatments: Bioassays were conducted in a controlled environment chamber at 23 ± 2 ℃, 60 ± 10 % RH, and 14/10 h (L/D) photoperiod. Leaf disks were placed individually into a transparent plastic Petri dish (9 cm in diameter) over moistened filter paper and one third instar larvae (aged 10 days) was added per dish (experimental unit). Larval survival was monitored every 24 h during three days. The consumed area of the disk was estimated using CobCal® program. Twenty replicates were performed for each treatment. To estimate the efficacy of each product against *T. absoluta* larvae, Abbott formulae was used[16].

Statistical analysis; To determine the effectiveness for each compound against *T. absoluta*, the leaf consumption of data obtained was subjected to a generalized linear model (GLM) with a normal distribution, type III error, and log link function at α= 0.05. Means were segregated using multiple pairwise comparison through Šidáktest (α= 0.05). *T. absoluta* longevity data was subjected to a Kaplan–Meier survival analysis (SAS 2008). Afterwards, a log-rank (Mantel–Cox) test was used to determine larval survival differences among treatments.

Acute toxicity on the crustacean species *Daphnia magna*: The most active aryl pyrazole (3c) against *T. absoluta* was tested on *Daphnia magna* according to the protocol described in OECD 202 guidelines for testing toxicity of chemicals products in the Laboratory of Technology of Uruguay (LATU)[17].

Young female *Daphnia*, aged less than 24 h, was exposed to the test substance and added to water with concentrations ranging from 0.3020 mg/L to 0.1020 mg/L. Specifically, 4 replicates of 10 individuals each were exposed individually to a concentration. Test temperature ranged between 18 and 22 °C and photoperiod was 16 h light. The test lasted 21 days, after which the total number of living offspring were assessed. The toxic effect of the test substance was expressed as EC_{50} by fitting the data to an appropiate model by non-linear regression[17].

Results and Discussion
Fipronil (T5) achieved the highest efficacy soon after 24 h followed by T3 and T2. T2, T1 and T4 differed significantly from Fipronil while T2 and T3 differed significantly from

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the least effective treatments, T1 and T4 (Figure 3). Statistically, the GLM reported significant differences among the different treatments regarding both efficacy (χ^2= 55.57; gl: 4.95; $p<0.01$) and tomato leaf consumption (χ^2= 41.37; df: 5.95; $p<0.01$).

Treatments T3 and T2 followed Fipronil in effectiveness, which is noteworthy since they are chemically obtained by a simpler route than that of Fipronil. T3 killed 75 % of the larvae, while T2 killed 60 % in 48 h. Fipronil also prevented *T. absoluta* larvae leaf consumption, while T3 and T2 showed a significant reduction in tomato leaf damage. T1 and T4 did not differ from the control treatment (Figure 4).
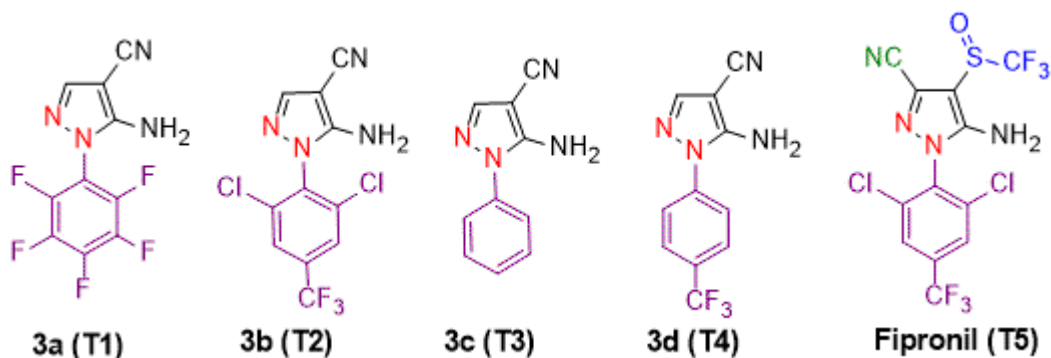

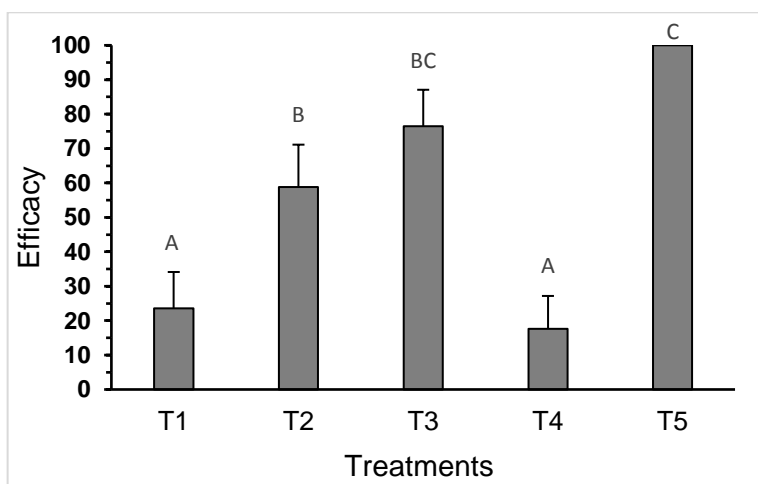**Figure 2: Structures of the synthesized N-aryl pyrazoles (3a-d) and Fipronil (T5)**

Figure 3: Efficacy ($\bar{X} \pm SE$) of 5 treatments on the control of *T.absoluta* after 48h of exposition. Bars crowned by the same letter indicate no significant differences (Šidák correction test $\alpha = 0.05$). T1 (3a), T2 (3b), T3 (3c), T4 (3d) and T5 (Fipronil).
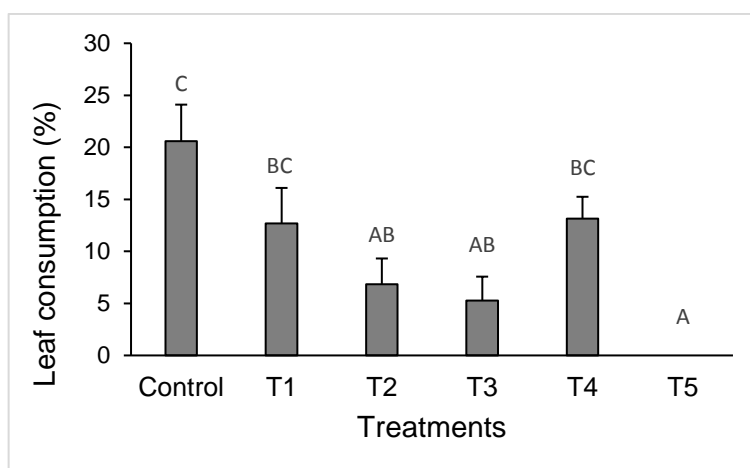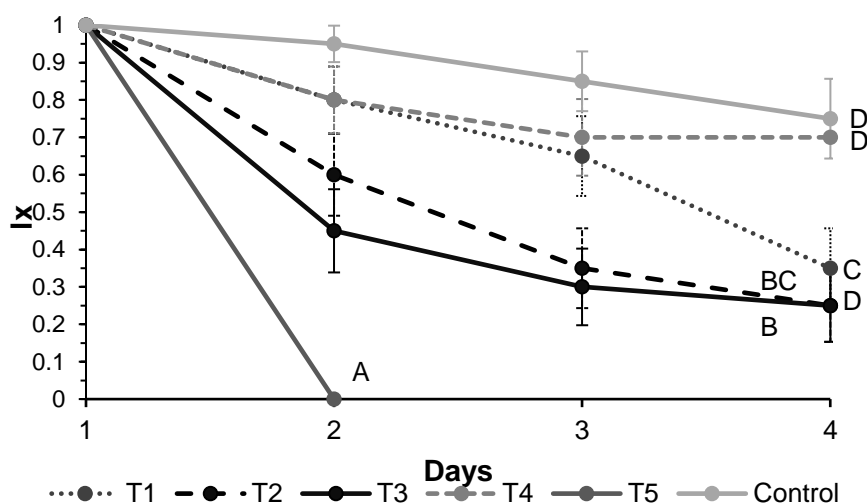

Figure 4: Percentage ($\bar{X} \pm SE$) of leaf damaged and/or consumed leaf area after 48 h of exposition to *T. absoluta* larvae.Control treatment corresponded to solvent alone, T1 (3a), T2 (3b), T3 (3c), T4 (3d) and T5 (Fipronil).

Figure 5: Larval survival ($\bar{X} \pm SE$) of *T. absoluta* after 72 h. Different letters denote significant differences, Log Rank (Mantel–Cox) test ($p<0.05$)

Fipronil acts by contact but also by ingestion[18]. Since the larvae did not consume the treated leafdiscks, the effect observed was attributed only to a contact effect. Statistic Kaplan–Meier analysis showed that larval survival was affected by the treatments ($\chi^2 = 52.26$; df: 5.115; $p<0.01$). Fipronil, T2 and T3 significantly reduced larval survival with respect to control. Larval survival for T4, T1, and control treatment (T6) was similar. The average survival ($\bar{X} \pm SE$) of the larvae in days was: 1.00 ± 0.00 for T5, 2.00 ± 0.28 for T3, 2.20 ± 0.27 for T2, 2.80 ± 0.25 for T1, 3.20 ± 0.28 for T4 and 3.45 ± 0.19 for the control treatment (Figure 5).

Compound 3c was further investigated on toxicity towards the crustacean species *D. magna*. Acute toxicity assessment on *D. magna* (EC_{50} 48 h) showed that the compound tested was non toxic until 1,020.0 mg/L. Compared to commercial Fipronil, which exhibited acute toxicity of 0.19 – 3.8 mg/L on daphnids[19], 3c proved to be much less toxic. The efficacy and mortality caused by Fipronil (T5) in *T. absoluta* larvae observed in the present study agree with its broad spectrum of action and effectiveness observed for other pests[18].

Compound 3b is structurally similar to Fipronil, as it presents two atoms of chlorine and a trifluoromethyl (-CF$_3$) group in the aromatic ring. Being Fipronil a non-competitive GABA receptor antagonist, 3b is expected to act similarly. The insecticidal activity of Fipronil is atributed to the aryl hydrazine that conforms this structure known as "magic aryl hydrazine" due to the presence of halogen atoms and the trifluoromethyl group[20].

Compounds 3b and 3c showed a similar insecticidal activity. The structure of 3c has no substituents on the pyrazole ring and it was synthesized with high yield (93 %) by a simple process much more green and economical than Fipronil. Finally, the N-aryl pyrazol 3c could be considered a promising alternative for pest control due to it low toxicity (*D. magna*) and high efficiency against *T. absoluta.*

Conclusion
It can be concluded that 5-amino-1-aryl-1*H*-pyrazole-4-carbonitriles (3a-d) were prepared following one-pot reaction with moderate to good yields. These N-aryl pyrazoles have the ability to preserve tomato crops because of their insecticidal activity. The efficiency of a preservative substance is reflected to a large extent in the resistance to insect growth, especially in the *T. absoluta* larvae that colonize the tomato leafs. Thus, 3b and 3c showed to have these properties when they were compared with biocide Fipronil.

Compound 3c was less toxic than Fipronil for *Daphnia magna* as a biological model and 3c was found to be a more potent insecticide in comparison with 3a, 3b and 3d respectively. In this line, the development of insecticidal agents from novel N-aryl pyrazoles seems to continue to be of great interest for both the academic and the industrial community, as it is necessary to develop less harmful and more selective compounds in order to protect the environment.

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