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Canavalia gladiata and *Dolichos lablab* extracts for sustainable pest biocontrol and plant nutrition improvement in El Salvador

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Abstract
Botanical repellents and pesticides are now being rediscovered as new tools for integrated pest management in order to reduce the use of toxic chemicals in crop production. *Canavalia gladiata* and *Dolichos lablab* are two Fabaceae very well adapted to farmlands of El Salvador, effective as living barriers and mostly as cover crops, however, they are not yet very well disseminated. This document describes the potential for using the liquid extracts and the dry flour of raw seeds of those plants for economic benefit and practical convenience for pest management in Salvadorian agriculture under field conditions. Seed extracts were useful when applied to the foliage to repel white flies (Aleyrodidae spp.) at the dose of 10% v/v; the repellent effect lasted approximately up to 8 days. The flour produced from the ground seeds were effective for preventing infections of Meloidogyne spp. and *Phyllophaga* spp., when mixed with soil prior to transplant; the beneficial effect lasted for about one month. Another added value of those flours was the contribution to plant nutrition in the short term, yet applying this treatment must be delayed until after crop germination and emergence because it can cause growth disorder in young seedlings. These leguminous crop seed extracts and flours appear to have promise for commercial application, especially by limited resource farmers

Keywords: Sugar cane, canavalia, dolichos, botanic pesticide, pest repellence, organic agriculture

1. Introduction
With the intention of reducing pest populations in soils and foliage, this research explored the use of natural extracts of *Canavalia gladiata* and *Dolichos lablab* to test their repellent effects and other possible contributions to plant nutrition. Arim *et al*. (2006) [4] conducted a study of the effects of using *Canavalia ensiformis* (very similar to *Canavalia gladiata*) as green manure, in the context of subsistence agriculture. A nematode reduction of *Pratylenchus* spp. up to 70% was shown in maize fields, also increasing yield because of nitrogen fixed by the cover crop. Follmer *et al*. (2001) [19] had already described the active ingredient as "canatoxin", a compound 86% similar to urease, which has insecticidal effects and it is lethal to mice at 2 mg/Kg. Carlini *et al*. (1997) [9] demonstrated that it is lethal for insects displaying cathepsin-based digestion at 0.25% wt, wt, affecting harmful insects like *Phyllophaga* spp. (Kuipers *et al*. (2004). *Canavalia gladiata* is very similar to *C. ensiformis* not only in their appearance but also in its toxicity potential. Ekanayake *et al*. (2007) [15] discovered more than one chemical with insecticidal effects on mature *Canavalia* beans, previously soaked for a few hours and subsequently squeezed to obtain a natural substance rich in "antinutritional factors such as haemagglutinins (concanavalin A), protease inhibitors (Laurena *et al*., 1994) [29, 30], hydrocyanic acid, tannins, phytates and canavanine (Kay, 1979) [28].
Dolichos bean had the highest trypsin inhibitor activity ranging from 14 to 27 units/mg (Laurena *et al*., 1994) [29, 30], which consists of a powerful agent that helps to avoid the attack of many kind of insects. Barbehenn *et al*. (2011) [5] described that in the first 5 hours when moisture starts the germination process in *Dolichos*, the highest concentrations of tannins are reached, increasing toxicity for hervibore pests (Osman, 2007). The reason those tannins are so useful for bio-protecting the crop is that "tannins are especially prone to oxidize in insects with high pH guts, forming semiquinone radicals and quinones, as well as other reactive oxygen species. Tannin toxicity in insects is thought to result from the production of high levels of reactive oxygen species" (Barbehenn *et al*., 2011) [5]. According to Janarthanan *et al*. (2008) [24] extracts of raw seeds of *Dolichos* contain high amounts of proteins similar to isoforms of arcelins 3 and 4 and pathogenesis-related protein 1 (PvPR1), causing the complete death of

Coleoptera: *Callosobruchus maculatus*, at doses of 5% of its regular diet. Those experiments prove that *Dolichos* raw beans contain more than one kind of toxic substance, all of them capable of controling different types of pests and with possible synergistic effect between them.

2. Methods

2.1 Production of raw material: For a first phase, half hectare for each legume, *Canavalia* and *Dolichos,* was planted in a well-drained silty loam soil with some stoniness and low fertility. The plantations were stablished at open sky and without the use of agrochemicals. Seeds proceeded from self-owned cultivars and received no prior treatment.

2.2 Natural extracts obtaining

As literature suggests, seeds are the part of the plant where most of the toxins are contained and their concentration is increased a few hours after exposing to moisture (Barbehenn *et al*., 2011; Ekanayake *et al*., 2007) [5, 15], reason for which they were immersed in water until swelling point and then compressed to separate solids from liquids. The extrusion process is efficient, for example it can obtain up to 90% of the essential oils of soybeans (Bargale *et al*., 1999) [6], whose structure is similar to the legume seeds used in this study. Extrusion offers a convenient method for tissue disruption due to strong compression forces and slight heating in a fraction of time, both in a single-step operation. This treatment contributes greatly to the retention of the natural characteristics of the natural substances while avoiding activation of damaging enzymes before they adversely affect essential oil quality (Nelson *et al*. 1987) [37]. Soetaredjo *et al*., (2008) [50] conducted a similar experiment using dry neem seeds in order to obtain oil for pest bio control, arriving at two main conclusions: pressure should be at least of 3000 psi (5000 was optimum for neem), and no pre heating of seeds was required. For the present study, seeds were collected and cleaned with an air flow at atmospheric temperature, oil was extracted with a 20 Ton hydraulic jack, which extruded seeds trough a recycled engine cylinder of 4 inches diameter, previously perforated with 12 holes of 4 milimeters diameter, creating a presion of 10,000 psi which exceeded the minimum required. This simple machine was a low cost adaptation of the very well-known principle of a hydraulic press.

Additionally, flours of these seeds were used to conduct additional tests including plant nutrition effect. Seeds of each legume were water soaked during a period of 24 hours, after which were grinded to obtain a natural paste, then it was sun dried and finally grinded again to produce fine flour.

2.3 Expected products

In case of *Canavalia*, the compound of major interest is canatoxin, with a concentration of 3.4% of the raw essential oil (Stanisçuaski *et al*., 2005; Oliveira *et al*., 1999; Carlini *et al*., 1997) [54, 9, 38]; additionally, each gram of raw seed contains: 4.8 mg of phytic acid, 2.4 mg of tannic acid and 22 µg of extremely poisonous Hydrogen cyanide (Laurena *et al*. 1994). [29, 30] The extract of the second legume, *Dolichos*, has an average tannin content of 2% (Deka, 1990) [14] and different amounts of anti-nutritional factors: dolichin (Ye *et al*., 2000) [58], trypsin inhibitor (Osman, 2007) [40], polyphenols and phytic acid (Ramakrishna *et al*., 2006) [44]. These last kinds of chemicals are enzyme inhibitors which are difficult to be measured in absolute terms (e.g. grams), because their purity tends to be low and a proportion of them could be inactive, a reason for which they are often designated as Inhibitory Units per gram (IU/g). Ramakrishna *et al*. (2006) [44] concluded that raw seeds of *Dolichos* (after 24 hours of soaking) have an average of 1916 trypsin IU/g, 82 phytic acid IU/g, and 3.5 total polyphenols IU/g.

2.4 Experimental design

For the present study all tests were performed with young plants in order to determine beneficial and adverse effects during the period in which crops are most susceptible. Biological compounds extracted from *Canavalia gladiata* and *Dolichos lablab* were simply named as *Canavalia* and *Dolichos* in the thesis. Their extracts were administered at different concentrations to be applied over the foliage of tomato, red beans and cucumber plants in order to test their repellent effects white fly. Other treatments were drenched on rice, tomato and cucumber plants, in order to evaluate their contribution to plant nutrition and the potential for repelling of *Phyllophaga*. The goal was to measure whether one dose for each treatment would be beneficial enough to contribute to the control of each specific pest problem. Tests were applied to two families of crops in one repetition for getting exploratory results, except for the Meloidogyne and plant nutrition experiments, in which a single crop was chosen to be tested in two repetitions. Several measurements like pest populations and plant growth were recorded periodically according to the particular conditions of each experiment, subsequently explained in this document. Bidimensional matrices were built for comparing results of treatment by plant families, and later confirmed with a post-hoc Tukey test to determine the significance of differences among the treatments and also comparing them to the controls. Considering that there were no repetitions for those experiments, a specialized statistical software was used to bootstrap the input data for increasing reliability of the results. Using this analysis, the dose with "more difference" compared to the control treatments was considered as the more adequate for practical uses, also including the criteria of selecting the lowest concentration to recommend for those cases with similar results.

To test repellence against *Meloidogyne* and beneficial effects on plant nutrition, treatments were directly applied to soil in the root zone of tomato plants during transplant. Two repetitions were included for a better statistical analysis of both experiments. Also, as organic matter from treatments was expected to decompose and release nutrients in soil, another experiment with tomato at the same doses of treatment was run in two repetitions to observe the effects on plant development. Each dose of all treatments was tabulated as an individual data series for regression analyses and obtaining of the correlation index between repetitions.

In detail, each of the mentioned experiments were performed as follow:

2.5. a) Repellence of *Phyllophaga*

Seven hundred *Phyllophaga* insects were collected from an infected rice field, their identification and collection were easy because of the symptoms of the plants that were being parasitized. Larvae were deposited in plastic bags filled with half kilogram of ground. Each bag contained one *Phyllophaga* and was located at open field, previously perforating 4 small holes for drainage. The reason for using plastic bags is that the pest is able to move underground to avoid unfavorable conditions, making it difficult to determine if individuals would die after treatment or just be repelled. The bags were classified in 14 groups of 50 units, to test six different treatments of Canavalia, six of Dolichos and two controls. Half of each bag group was transplanted with rice and the other with cucumber plants. The doses for each treatment were 3 grams, 6 grams and 12 grams of Canavalia and Dolichos. One of the control treatments contained no flour and the other consisted on 6 grams of corn starch. After 8 days, bags were opened in order to observe the death or repellence of the insect. In the second phase of the experiment, the whole experiment was repeated but with the difference that bags were not opened until the activity of the insects reappeared, to evaluate the possible loss of the repellent effects.

2.5. b) Repellence of white flies

Seven hundred red bean plants were planted during the dry season when the incidence of this pest tends to be highest. Fifteen days after germination, the foliage of every hundred plants were treated as described in the previous experiment (3.6.a). Tomato was the selected crop due to its susceptibility to white fly. The number of insects was determined using the following procedure: a leaf was gently held between two pieces of transparent acrylic sheet and then cut off the plant, a square centimeter was selected as a sample and the adults found inside were counted. This procedure was repeated with one another plant. Data was obtained at two different times for comparison purposes: two days after treatment and seven days after treatment.

2.5. c) Repellence of *Meloidogyne*.

A *Meloidogyne* culture was developed in a local laboratory in order to obtain a reliable pathogenic inoculum. The experiment was conducted with the same number of plastic bags and the same treatment schema as in the first phase of the experiment described in section 3.6.d. However, the differences were: the crop used for the tests was tomato and the whole experiment had two repetitions. Five nematode nodules of a previously infected host were situated aside the root zone, and the plants were fertilized with 4 grams of urea to increase the possibility of nematode infection by nitrogen stimulation. Plastic bags were opened for root examination after 6 weeks from transplant. Nodulation was visually recognized using the method of Weighted Nematode Rating (UC-IPM, 2004; Flint, 2012) [53, 18] in which index 0 is assigned when 0% of root system exhibits galling, index 1 is for damage ranging from 1% to 25%, 3 for 26% to 50%, 5 for 51% to 75 % and 7 for 76% to 100%.

2.5. d) Effects of plant nutrition.

Repelled Dead No effect

The experiment was conducted with the same treatment schema as the first phase of the experiment described in Section 3.6.d. However, in this experiment tomato seedlings were transplant to natural soil (instead of plastic bags) inside of a macro tunnel at day number 30 after germination. There were two repetitions. Treatments were applied at two moments: during transplant and two weeks after transplanting. Plant height was measured weekly every five days for statistical analysis.

3. Results

3.1 White flies repellence

White flies could be temporary controlled by the application of tested extracts on red beans and tomato crops. A 10% dose of Canavalia extract showed a good repellent effect on white flies within a week after application. The dose of 20% did not show a significant increase of effectiveness but did cause a counter effect by an apparent biochemical unbalance in foliage which was noticeable because the plants in those treatments stopped growing during that period of time. Such a side effect was less pronounced in Dolichos treatments, which showed an acceptable repellent effect at the dose of 20%. Tukey tests demonstrated that the biggest significant difference in insect populations is at 10% and 20% concentration with respect to the control for both extracts tested, the mean difference is big enough to support that assertion and the significance index indicates quantitative proof of this difference. The Pearson correlation obtained by comparing the data results of Canavalia extracts on red beans vs tomato was r2 = 0.976, significant at the 0.01 level with a 95% confidence interval, run on a model of 1000 bootstrap samples. The high level of correlation indicate that the results of the treatments can be considered equivalent among the two different crops, although they were in adjacent fields and not truly replicated. In the case of Dolichos treatments, correlation results were similar, with a value of r2 = 0.950.

3.2 *Phyllophaga* repellence

Canavalia flour had a strong repellent effect on *Phyllophaga* spp. While both control treatments were seriously affected by this pest, most of the plants provided with this treatment could be kept safe for about a month. This effect was similar for rice and cucumber crops, along with the observation of improved plant vigor as the dose was increased. About half of the pest individuals died after the application. Survival was measured in a separate experiment a month after treatment; it was found that about half of the surviving pests were able to reinfest crop plants. In the case of Dolichos treatments, results were proportional but less intense. Graphs in Figure 1 show that in the case of *Canavalia* treatment, almost all pest individuals could be controlled with either dose. *Dolichos* treatment proved to be relatively less effective and its control power was proportional to the dose provided. Tukey tests demonstrated that there is a significant difference between the *Phyllophaga* populations at every concentration of *Canavalia* flour. The mean difference is big enough to support that assertion and the significance index indicates quantitative proof. The Pearson correlation obtained by comparing the data results of *Canavalia* extracts on red beans vs tomato, was $r^2 = 0.987$, significant at the 0.01 level with a 95%

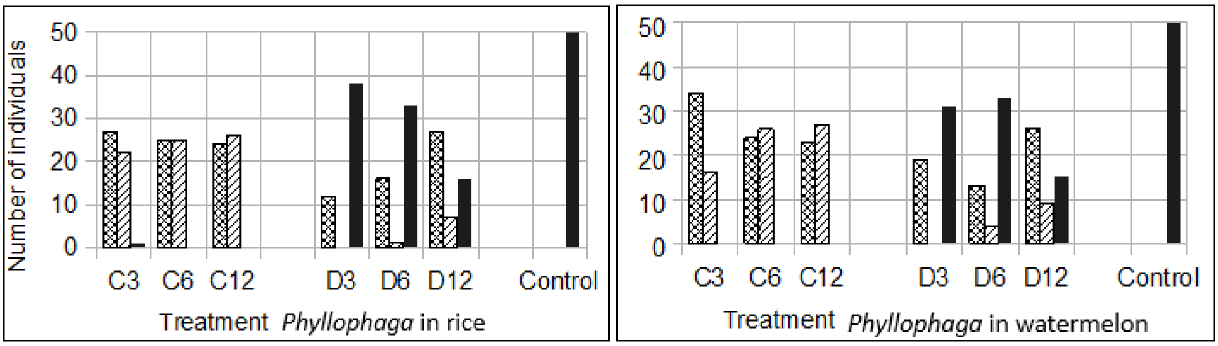

Fig 1: Count of *Phyllophaga* individuals in rice (A) and watermelon (B) plants, treated with three different concentrations of Canavalia and Dolichos flours and then compared with two "Control" treatments of identical results: corn starch and no bio chemical added. In the labels of X axis, letter "C" stands for Canavalia treatment and "D" for Dolichos; the contiguous numbers of 3, 6 and 12, indicate the amount of grams of flour per treatment.

Confidence interval, run on a model of 1000 bootstrap samples. The high level of correlation indicate that the results of the treatments can be considered equivalent between the two crops. In the case of Dolichos treatments, correlation result was similar, with a value of r2 = 0.984 (significant at the 0.01 level).

3.3 Nematode repellence

Disease caused by *Meloidogyne* was significantly reduced by the addition of 6 grams of *Canavalia* flour in tomato root zones at the time of transplant. The dose of 12 grams did not exhibit a great change on disease prevention but it provided an apparent increased foliage vigor to the plant.

For *Dolichos* treatments, 12 g dose was the more effective but still exhibited less advantages compared *to Canavalia* (Figure 2). The inoculated control plants proved that the infection method was successful and the non-inoculated control eliminates other variables as the cause of differences between treatments. The coefficient of correlation of the data obtained during the first and second repetition, were 0.99 for *Canavalia* treatments and 0.98 for *Dolichos treatments,* leading to the assumption that the resulting averages were in concordance.

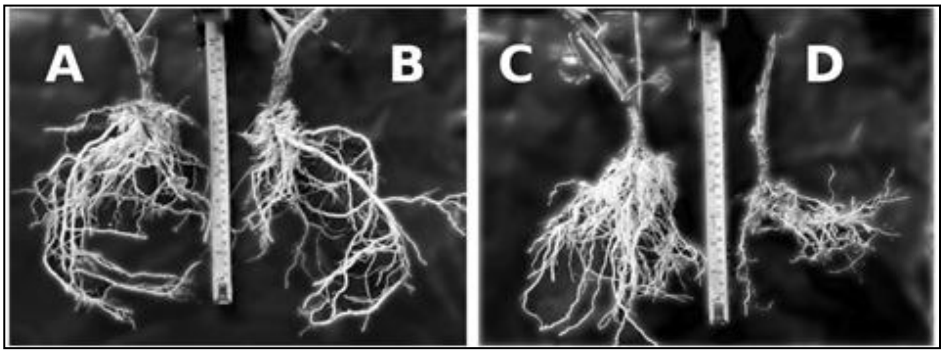

Fig 2: Tomato roots, 30 days after infestation with *Meloidogyne*. Central measurement tape in both pictures is indicating 10.5 inches, for visual reference. Roots treated with (A) X mg of *Canavalia* flour manifested a mild level disease, (B) same treatment with *Dolichos* exhibited a moderate level disease, (C) a non-inoculated healthy root system served for control and (D) another successfully infected plant with no treatment showed a severe disease damage. *Photos: Carlos Martínez.*

3.4 Effects on plant nutrition

Soil analysis revealed that the soil were experiments took place had a pH of 4.6, 3.5% of organic matter, high availability of Mg and Fe, intermediate availability of K, Ca, S, Mo, Cu, Mn, and very low levels of P, B, Zn, N. According to the composition of *Canavalia* and *Dolichos* flours, it was estimated that their major contributions to plants were nitrogen, boron, phosphorus, potassium and sulfur (Table 1). Doses of 3 grams of each flour per plant showed less effective results than the dose of 6 grams. However, the application of 12 grams did not show any undesired side effect, but also showed an important advantage over its predecessor because the extra amount of matter had a slower decomposition rate; its nutrients would be beneficial in a longer period of time than the lapse of 50 days considered in this study, meaning that 6 grams per plant would be a more suitable dose for results in the short term.

Figure 2 compares the results of treatment after first repetition and Figure 3, the second repetition. There were differences on the growth pattern of the subject plants because environmental conditions were hotter and dryer during the first test than in the second. However, correlation indexes between repetitions were 0.992 for *Canavalia* treatments, 0.997 for *Dolichos*, 0.938 for Control 1 (corn starch) and 0.948 for Control 2 (no extra treatment). Both treatments proved to have beneficial effects on plant nutrition; the reason because *Dolichos* exhibited more beneficial effects could be related to the fact that its decomposition seemed to be faster.

Table 1: Mineral nutrient content of flours

Nutrient	Canavalia flour	Dolichos flour	Method
N	2.83 %	2.48 %	Kjeldahl
P	0.07 %	0.07 %	Colorimetry
K	0.89 %	0.65 %	Emission spectrophotometry

Ca	0.08 %	0.06 %	AA spectrophotometry
Mg	0.10 %	0.11 %	AA spectrophotometry
S	0.06 %	0.37 %	Colorimetry
Fe	0.02 %	0.02 %	AA spectrophotometry
Mn	0.00 %	0.00 %	AA spectrophotometry
Zn	0.00 %	0.00 %	AA spectrophotometry
B	0.23 %	0.30 %	Colorimetry
Cu	0.00 %	0.00%	AA spectrophotometry

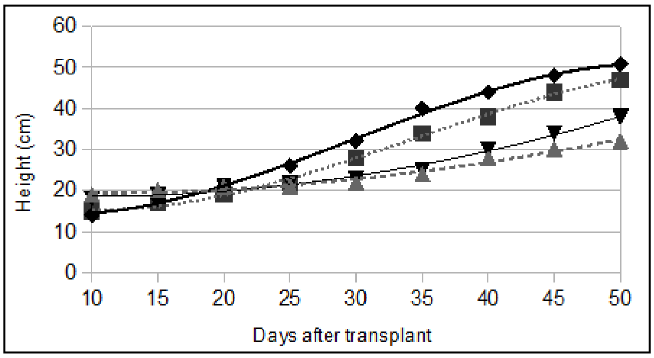

Fig 3: Comparison of the growth of tomato plants in four treatments, during the first repetition of the experiment. The dose of 6 grams of *Canavalia, Dolichos* and corn starch (Control 1) were 6 grams of dry flour. Control 2 was provided with no flour. Points represent the average of each group and the regression line for *Canavalia* was *f(x)* $-0.081x^3 + 1.77x^2 - 5.99x + 21.3$; for Dolichos, $f(x) = -0.101x^3 + 1.81x^2 - 4.68x + 17.4$; for Control 1, $f(x) = 0.026x^3 + 0.175x^2 - 1.41x + 15.8$ and for Control 2: $f(x) = -0.005x^3 + 0.287x^2 - 1.19x + 20.7$.

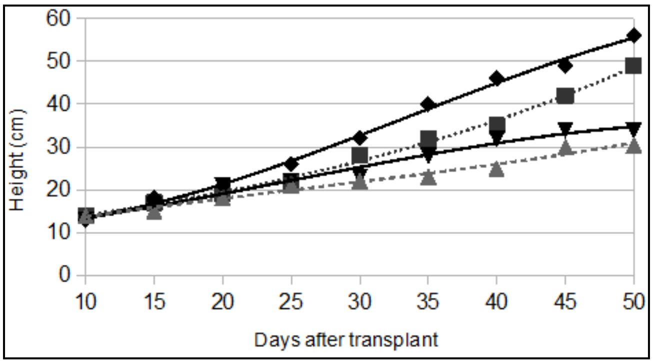

Fig 4: Comparison of the growth of tomato plants in four treatments, during the second repetition of the experiment. The dose of 6 grams of *Canavalia, Dolichos* and corn starch (Control 1) were 6 grams of dry flour. Control 2 was provided with no flour. Points represent the average of each group and the regression line for *Canavalia* is $f(x) = -0.014x^3 + 0.74x^2 - 2.30x + 11.6$; for *Dolichos*, $f(x) = -0.055x^3 + 0.945x^2 - 0.853x + 11.7$; for Control 1, $f(x) = 0.024x^3 + 0.293x^2 - 1.96x + 11.2$ and for Control 2, $f(x) = -0.013x^3 + 0.169x^2 - 2.66x + 11.1$

3.5 Other non-measured observations

There were other phenomena that occurred unexpectedly and were not properly measured because they were not part of the experiment design. However, these 'emergent properties' could build interest in the topic for future research, the reason for which they are briefly mentioned in this section. The first was the observation of a certain repellent effect on lepidoptera in both treatments, *Canavalia* and *Dolichos*. A second situation was that several tests on soil had to be repeated due to the development of ecto mycorhizae (under field conditions), especially in those treatments with 6 and 12 grams of Canavalia flour, suggesting that the substance was not antagonist to this symbiotic microorganism and yet favored the infection. One last finding was that flours applied over the soil surface seemed to repel defoliation by ants and also tended to benefit inflorescence development.

4. Discussion

An important number of white flies (tested on cucumber and tomato) could be repelled for a period of seven days with *Canavalia* treatment at the dose of 10%. In contrast, 20% concentration showed only little improvement and caused a dark green leaf coloration with plant stunting for an approximate period of one week; development was later resumed and plants could eventually reach the same size as Control plants. *Dolichos* showed a more limited repellence effect with the same temporal plant stunting. However, this results may not be beneficial enough since white flies are vectors of virus and the reduction on the population of this pest cannot completely guarantee the absence of infestation. For this reason, preventive treatmentss with *Canavalia* at the concentration of 5% are preferable than corrective treatments. *Phyllophaga*, could be effectively repelled by *Canavalia* treatment. However, *Dolichos* exhibited a slightly weaker effect. About half of the insects died and the survivors could resume their parasitic activity after a month. The presence of increased organic matter in soil must not be considered the cause, because the corn starch control treatment exhibited a zero repellence activity. In other terms, the *Canavalia* and *Dolichos* flours applied to soil in the previous moment to transplant did not exhibit any kind of counterproductive effect. In contrast, they appeared to have a beneficial influence on plant nutrition. These results are in concordance with Pino *et al*. (2013) [42] and Meseguer *et al*. (2008) [33] who described that the powder of a species of *Canavalia* possesses an effective insecticidal and repellent effect over *Sitophilus zeamais* Motschulsky, which is a Coleptera similar to the *Phyllophaga* tested in this study. Other control effects could be found in literature in order to compare the results of this study: a insecticidal peptide derived from a *Canavalia* species, which was convenient for control of *Spodoptera frugiperda* and *Dysdercus peruvianus* (Mulinari *et al*., 2007) [35], toxicity for *Oncopeltus fasciatus* (Defferrari *et al*., 2011) [13], Atta sexdens (Hebling *et al*., 2000) [22] and Acyrthosiphon pisum (Sauvion *et al*., 2004) [47]. Results from some experiments indicate that *Dolichos* have a real but limited insecticidal/repellent effect. For example, *Dolichos* has been reported as adulticidal for many *Diptera* (Kamaraj *et al*., 2010; Hazarika *et al*., 2012; Mavundza *et al*., 2014) [25, 21, 32], and toxic to some *Adisura atkinsoni* at the dose of 10% v/v (Chakravarthy *et al*., 1985) [11], *Canavalia gladiata* flour demonstrated a reduced infestation of *Meloidogyne*, as it has been previously demostrated for *Canavalia ensiformis* (Lopes *et al*., 2009; Crozzoli *et al*., 2001) [31, 12], which is capable to produce *"oxidation of the cuticle with periodate under mild conditions"* (Spiegel *et al*., 2011) [53]. *Dolichos lablab* flour showed a certain suppression level for *Meloidogyne* damage, similarly as proven in laboratory conditions (Araya, *et al*., 1994) [2], or in open field when the foliar biomass is incorporated as cover crop (Brandenburg *et al*., 2010) [39]. In all experiments, corn starch had a non-relevant beneficial effect, indicating that the positive results of *Canavalia* and *Dolichos* flours cannot be achieved by every kind of organic substances. The ground powders of the these seeds possess a useful effect for integrated pest management programs and can significantly contribute to plant nutrition in the short term, which is comparable to the evidence of those cases in which soil fertility has been restored by the incorporation of cover crops of a similar species, *Canavalia ensiformis* (Pohlan *et al*., 2008; Fageria *et al*., 2005; Buckles *et al*., 1998) [43, 17, 8] and also *Dolichos lablab* (Karuma *et al*., 2011; Carsky *et al*., 2001; Schaaffhausen, 1963) [27, 10, 48]

5. Conclusions

Canavalia gladiata and *Dolichos lablab* are two legumes that can be used to improve soil fertility. In subsistence agriculture, this practice is particularly recommended because of its low cost and the possibility of using raw seeds to produce botanical insecticides or repellents that contribute to the organic production of vegetables and cereals. Their extracts can repel some foliage insects and the flour of their ground seeds applied to soil can contribute to preventing some Coleptera pests, reducing the need of dangerous agrochemicals. Canavalia exhibited more benefits than Dolichos in the case of Salvadorian agriculture. Integrated nematode management plans can also be improved by the addition of these types of flour in the root zones during the transplant, also contributing to plant nutrition as the organic matter is decomposed. All the mentioned treatments are recommended for plants that have already past the emergence phase, in order to avoid negative side effects of the applied flour from these two legumes.

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