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# Germination and root nodule formation of soybean (*Glycine max* (L.) Merr.) in ridomil and chlorpyriphos treated soil

**Ibiang Young Bassey[1, *], Ekanem Bassey Effiong[1, 2], Usanga Daniel Archibong[1], Williams Uduak Ita[1]**

[1]Department of Genetics and Biotechnology, University of Calabar, PMB 1115, Calabar, Nigeria
[2]Department of Science Technology, Akwa Ibom State Polytechnic Ikot Osurua, PMB 1200, Ikot Ekpene, Nigeria

### Email address:
youngangale@yahoo.com (Ibiang Y. B.), ekanembassey@yahoo.com (Ekanem B. E.), danielusanga@yahoo.com (Usanga D. A.), favourwilliams29@gmail.com (Williams U. I.)

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**Abstract:** Germination and root nodule formation of soybean (*Glycine max*) was evaluated in soil treated with Ridomil (R), Chlorpyriphos (CH), and a Combination of both (COM); using a completely randomized design, and a 3×3 factorial arrangement. Factor 1 was the pesticide type with 3 levels - R, CH, and COM; while factor 2 was the contamination rate (v/wt) with 3 levels - 0% (control), 2%, and 4%. Aqueous pesticides preparations (as recommended by manufacturers) were applied once, before seeds were planted. More than Ridomil and Combination, significantly ($P<0.05$) diminished percentage germination due to Chlorpyriphos was observed at 2, 3, and 4 weeks post application. The control had significantly higher germination than 2% and 4% groups, up until the 5th week. The Chlorpyriphos and pesticide Combination groups both had significantly lower root nodule number/plant, than Ridomil. Root nodule number/plant, and nodule biomass/plant were significantly reduced due to pesticide stress at 2% and 4%. Pesticides application at 2% and 4% negatively affected germination and root nodule formation; but there was no indication of an enhanced effect due to pesticides combination.

**Keywords:** Chlorpyriphos, Ridomil, Pesticide Interaction, Toxicity, Germination, Root Nodule, Soybean

## 1. Introduction

With the growing global concern about environmental sustainability, the impact of environmental stressors on the welfare of life forms and ecosystem processes remains a front burner in the intellectual and public domains. While environmental pollutants such as spilled crude oil, heavy metals, and toxic industrial effluents are widely regarded as stressors of living systems, Ashraf and Harris [1] describe plant “stress” to be any environmental factor that limits crop productivity or obtainable biomass, including drought, heat, cold, pathogens, salt, chemicals, etc [2]. Rapidly increasing human population, especially in developing countries, necessitates increased food and cash crop production, with attendant increase in utilization of farm inputs such as pesticides and fertilizers [3]. Despite the usefulness of pesticides in crop protection and food preservation, they have been associated with food poisoning, biological magnification, toxicity to non target species, and are agents of stress in the ecosystem. Further, pesticides use has not been without peculiar challenges such as high residues on food produce; the use of banned formulations; improper application procedures/precautions; inadequate agri-extension support and monitoring; soil and water pollution; amongst others [4, 5]. In Nigeria, cocoa farming contributes greatly to overall pesticides use, with formulations like Ridomil, deltamethrin, chlorpyriphos, etc, being popular with local farmers [6].

Aside toxicity to micro and meso-fauna [7], short and medium term environmental toxicity of pesticides have been illustrated in the form of oxidative stress in plant cells with reduced plant metabolism and growth, reduced nodulation, nitrogenase activity, as well as legume-rhizobium symbiosis, amongst others [8-11]. Other parameters such as germination, litter decomposition, soil enzyme levels, microbial counts/metabolic coefficients [12, 13], are influenced by chemical pesticides, and have a potential to be used as markers of environmental stress; although plants have generally not been utilized in environmental health monitoring as much as animals like fish and macroinvertebrate have. Soybean (*Glycine max* (L.) Merr.) is of ecological significance due to its role in enhancing nitrogen fixation by harbouring nitrogen fixing bacteria in its root nodules, as is typical of legumes [14].

While utilization of pesticides in line with manufacturer recommendations reduces the risk of harm due to their handling and application, chemical pesticides in use still remain an environmental hazard to be managed; and assessment within the scope of their local environmental releases/use patterns shed more light in this regard. Against the backdrop of pesticides drift and deposition, as well as dissolution in and adsorption to matter when they are applied, organisms could be exposed to more than one pesticide in the ecosystem, at any given locality. And interaction between two (or more) pesticides can occur, resulting in (enhanced) toxic effects much greater than is obtainable for equivalents of either pesticide alone [15]. Ridomil is a fungicide employed against the black pod disease, while chlorpyriphos is a broad spectrum insecticide used against mirids on cocoa. As a result, both pesticides do get to be used together in the same vicinity; taking into consideration the diversity of spraying regimen possible at farms. These form much of the underlying rationale for this study, using two ecologically significant processes; germination and root nodule formation, in the soybean plant. Having observed the paucity of literature reports on the combination of both pesticides, this study was carried out to evaluate the effect of Ridomil and Chlorpyriphos, singly and in combination, on germination as well as root nodule formation, in soybean; with a view to assessing the potential for enhanced effects, when both pesticides are used at rates recommended by the manufacturers.

# 2. Materials and Methods

### *2.1. Pesticides Preparations*

Ridomil Gold® 66WP (6% metalaxyl; 60% copper I oxide) (Syngenta Agro AG) and Chlorpyriphos 20% EC (Perfect Killer®, Nantong Jinling Agric Chem Co. Ltd, Jiangsu, China) were purchased from Egoob Chemicals, Watt market Calabar. Manufacturer recommended preparation of Ridomil for use on cocoa was 50g in 15 L of water; while that for Chlorpyriphos was 1250 ml in 500 L of water. These were stated on the pesticide labels, and adopted for the study. 1 L aqueous pesticides preparations were made for both pesticides, in accordance with the manufacturer recommendations (i.e. 3.33 g $L^{-1}$ for Ridomil; and 2.5 ml $L^{-1}$ for Chlorpyriphos), and applied immediately to respective experimental units.

### *2.2. Experimental Design and Treatment Groupings*

Employing a Completely randomized design, the experiment was laid out in a 3×3 factorial. Factor one was the pesticide type with three levels - Ridomil (R), Chlorpyriphos (CH), and Combination of both (COM); while factor two was the application/contamination rate (v/wt), also with three levels - 0% (control), 2% (20 ml of aqueous pesticide preparation $kg^{-1}$ of soil), and 4% (40 ml of aqueous pesticide preparation $kg^{-1}$ of soil). This gave a total of nine treatment combinations. 20 ml of pesticides combination was achieved thus: 10 ml of Ridomil preparation + 10 ml of Chlorpyriphos preparation $kg^{-1}$ of soil; while 40 ml of pesticides combination was achieved in similar vein i.e. 20 ml of Ridomil preparation + 20 ml of Chlorpyriphos preparation $kg^{-1}$ of soil). Three replicates were made for each treatment combination, giving a total of 27 experimental units.

### *2.3. Experimental Procedure*

Top soil was collected 0-20 cm deep at Biological Sciences Experimental Farm, University of Calabar, Calabar. The soil (5.3% clay; 6.7% silt; 87.2% sand; pH 6.03) was sieved using a wire mesh (<5mm) to remove stones and other debris, mixed thoroughly, and kept for one week on a spread-out polythene sheet. 1 kg of soil sample was weighed into labeled plastic containers, to which corresponding pesticide preparations were applied, once. Distilled water was added to ensure even liquid content in all vessels (plus control), and approximately 70% of maximum water holding capacity [12]; and mixed. After pesticides treatment, experimental units were kept in the open, exposed to natural daylight but protected from rainfall. After 24 hours, soybean (*G. max*) seeds were planted. Seeds were obtained from the germplasm collection of Department of Genetics and Biotechnology, University of Calabar, Calabar, and were of the same cultivar type; TGX 728-ID. Prior to planting, seeds were soaked in tap water for 5 minutes, and floating ones discarded [16]. 15 seeds were planted in each container, at a depth of 2cm. After 72 hours, soybean seeds were recovered for estimation of percentage germination (based on unequivocal radical emergence) [17]. With the exception of pesticide treatment, which was done once, seed planting and percentage germination was determined weekly, for 7 more weeks. Only distilled water (up to 70% holding capacity) was repeatedly added to soil (with re-mixing) in each container, once a week, exactly 24 hours prior to seed planting. For the root nodule study, 5 kg of soil sample was weighed into polythene earth bags with holes and polluted with respective pesticides preparations. Soybean seeds were planted and later thinned to 2 stands per bag. After three months the earth bags were carefully destroyed with a knife in a bucket of water, and number and dry biomass of root nodules (oven dried at 50°C until constant weight) was determined. In the same vein, pesticide treatment was performed once, while weekly addition of water was done at the same volume in all the experimental units. A plastic tray was placed under each earth bag and collected water (drain) was returned into the same earth bag.

$$\% \text{ Germination} = \text{No. of germinated seeds/Total no. of seeds planted} \times (100) \quad (1)$$

### 2.4. Statistical Analysis

All data generated were subjected to two-way analysis of variance (ANOVA) to check for significant differences between the treatment groups, and conclusions drawn at 5% probability level. Least significant difference (LSD) test was performed to separate means, where significant differences where noticed.

## 3. Results

### 3.1. Germination Rate

Table 1 show the effect of the pesticide types (Ridomil, Chlorpyriphos, and their Combination) on percentage germination of soybean. In the first week, no significant ($P>0.05$) differences were observed in percentage germination, between the pesticide types, but Chlorpyriphos had the lowest germination rate (54.07%) while the combination had the highest (61.47%). In the second week, significant ($P < 0.05$) differences were observed in the germination rates, with chlorpyriphos having the lowest value (57.77%) which differed significantly from that of Ridomil (69.62%) and combination (69.58%). In the third week, Ridomil and Combination had no difference between them for soybean germination rate. But their values, however, differed significantly from that of Chlorpyriphos, which had the lowest value. In the fourth week, Ridomil had a significantly higher value (67.40%) than Chlorpyriphos (55.55%) and Combination (60.73%). In the fifth to eight weeks there were no significant differences in germination rate, between the pesticide types.

***Table 1.*** *Effect of pesticide type on germination of soybeans (G. max).*

| Week | R | CH | COM | LSD |
|---|---|---|---|---|
| 1 | 57.03 ± 6.09 | 54.07 ± 6.23 | 61.47 ± 6.55 | NS |
| 2 | $69.62^{a}$± 2.96 | $57.77^{b}$ ± 6.47 | $69.58^{a}$ ± 1.73 | 8.33 |
| 3 | $69.55^{a}$ ± 3.33 | $58.51^{b}$ ± 6.36 | $69.62^{a}$ ± 3.86 | 10.2 |
| 4 | $67.40^{a}$ ± 2.59 | $55.55^{b}$ ± 4.44 | $60.73^{b}$ ±3.22 | 6.00 |
| 5 | 64.44 ± 4.15 | 67.40 ± 4.77 | 67.40 ± 3.91 | NS |
| 6 | 65.92 ± 3.03 | 68.14 ± 3.81 | 68.14 ± 2.42 | NS |
| 7 | 64.44 ± 2.93 | 65.25 ± 3.03 | 65.51 ± 3.09 | NS |
| 8 | 66.47 ± 2.89 | 65.95 ± 1.62 | 64.77 ± 3.68 | NS |

Values are presented as Mean ± SEM. $^{ab}$Values across the table with similar superscript are not significantly different at 5% based on ANOVA. NS = Not significant; R=Ridomil; CH = Chlorpyriphos; COM = Ridomil + Chlorpyriphos Combination.

Table 2 show the effect of pesticides concentration on germination of soybeans. In the first week, pesticide concentration showed dose dependent significant differences, with the control (0 ml $kg^{-1}$) having the highest percentage germination (75.55%), followed by 20 ml $kg^{-1}$ (58.51%) and 40 ml $kg^{-1}$ (38.51%), which had the lowest. All three concentrations differed significantly from one another. In the second and third week, the effect of pesticide concentration differed significantly in a dose-dependent manner, with the control having the highest germination rate and 40 ml $kg^{-1}$ having the lowest. In the fourth week, the control had significantly higher germination (78.88%) than both 20 ml $kg^{-1}$ (61.25%) and 40 ml $kg^{-1}$ (55.55%). Again this was the case in the fifth week, with the control having a significantly higher value than the other groups. From week six to eight, there were no significant differences in germination rate between the control and the other treatment groups. Nevertheless, control had higher values than 20 ml $kg^{-1}$ and 40 ml $kg^{-1}$ treatment groups.

***Table 2.*** *Effect of pesticide contamination rate on germination of soybean (G. max).*

| Week | 0 ml $kg^{-1}$ | 20 ml $kg^{-1}$ | 40 ml $kg^{-1}$ | LSD |
|---|---|---|---|---|
| 1 | $75.55^{a}$± 2.93 | $58.51^{b}$± 4.12 | $38.51^{c}$± 3.29 | 10.47 |
| 2 | $73.33^{a}$±1.92 | $68.36^{a}$ ±2.51 | $56.29^{b}$ ±6.19 | 8.33 |
| 3 | $79.99^{a}$ ± 3.84 | $64.44^{b}$ ± 5.44 | $59.25^{b}$ ±3.41 | 10.20 |
| 4 | $78.88^{a}$ ± 1.11 | $61.25^{b}$ ± 3.03 | $55.55^{b}$ ± 4.84 | 6.60 |
| 5 | $75.55^{a}$ ± 2.93 | $64.44^{ab}$± 3.51 | $59.25^{b}$ ± 4.22 | 11.98 |
| 6 | 73.33 ± 1.92 | 65.81 ± 3.64 | 63.7 ± 2.51 | NS |
| 7 | 76.66 ± 1.92 | 69.29 ±2.51 | 69.25 ± 3.59 | NS |
| 8 | 74.44 ± 1.11 | 69.51 ± 2.89 | 69.25 ± 3.75 | NS |

Values are presented as Mean ± SEM. $^{abc}$Values across the table with similar superscript are not significantly different at 5% based on ANOVA. NS = Not significant; 0ml $kg^{-1}$ = control; 20 ml $kg^{-1}$ = 2% (v/wt); 40 ml $kg^{-1}$ = 4% (v/wt).

### 3.2. Root nodule Formation

There were significant differences in root nodule number/plant between the pesticide types, with Ridomil having the highest number (6.00) while Chlorpyriphos had the lowest (4.77) (see Table 3). There were no significant differences in root nodule biomass/plant between the pesticide types, but the highest biomass was recorded in the Ridomil group (0.39g), while the lowest was recorded in the chlorpyriphos group (0.26g). Pesticides concentration had significant effects on root nodule number/plant (Table 4), with the control having a significantly higher number of root nodules (6.00) than 40 ml $kg^{-1}$ (4.44). 0 ml $kg^{-1}$ group (control) had significantly higher root nodule biomass than both 20 ml $kg^{-1}$ and 40 ml $kg^{-1}$ groups.

***Table 3.*** *Effect of pesticide type on root nodule formation in soybean (G. max).*

| Parameter | R | CH | COM | LSD |
|---|---|---|---|---|
| Root nodule number/plant | $6.00^{a}$± 0.23 | $4.77^{b}$±0.46 | $5.00^{b}$±0.46 | 0.95 |
| Root nodule biomass/plant (g) | 0.39± 0.04 | 0.26± 0.06 | 0.28±0.06 | NS |

Values are presented as Mean ± SEM. $^{ab}$Values across the table with similar superscript are not significantly different at 5% based on ANOVA. NS = Not significant; R=Ridomil; CH = Chlorpyriphos; COM = Ridomil + Chlorpyriphos Combination.

***Table 4.*** *Effect of pesticide contamination rate on root nodule formation in soybean (G. max).*

| Parameter | 0 ml kg$^{-1}$ | 20 ml kg$^{-1}$ | 40 ml kg$^{-1}$ | LSD |
|---|---|---|---|---|
| Root nodule number/ plant | $6.00^{a} \pm 0.28$ | $5.33^{ab} \pm 0.40$ | $4.44^{b} \pm 0.37$ | 0.95 |
| Root nodule biomass/plant (g) | $0.49^{a} \pm 0.04$ | $0.24^{b} \pm 0.03$ | $0.21^{b} \pm 0.05$ | 0.13 |

Values are presented as Mean $\pm$ SEM. [ab]Values across the table with similar superscript are not significantly different at 5% based on ANOVA. NS = Not significant; 0ml kg$^{-1}$ = control; 20 ml kg$^{-1}$ = 2% (v/wt); 40 ml kg$^{-1}$ = 4% (v/wt).

## 4. Discussion

Results obtained showed that the pesticides inhibited seed germination of soybean, as well as development of root nodules. Between the pesticide types, chlorpyriphos had the lowest seed germination, while Ridomil and the Combination were comparables; with the significant effect occurring from weeks 2 to 4. This implies that as recommended for use on cocoa by the manufacturers, chlorpyriphos is a “higher” stressor to soybean seeds than Ridomil. This is in line with the position of Dubey and Fulekar [18] that chlorpyriphos contamination in soil reduces germination rate of some plants. No significant differences between the pesticide types were observed in the first week (Table 1). This perhaps, is because all pesticide types were nearly equally inhibitory to germination in the first week (very low germination rates in week one of Table 2, for both 20 ml kg$^{-1}$ and 40 ml kg$^{-1}$ supports this). But by the second – fourth weeks, chlorpyriphos retained the highest inhibitory effect (below 50%) on soybean seeds germination. Overall, the seeds in control soils had significantly higher germination rates than those in soils treated with 20 ml kg$^{-1}$ and 40 ml kg$^{-1}$ of pesticides (Table 2) up until the fifth week. This highlights the (short term) stressful effects both pesticides had on soybean seed germination, at 2% and 4% concentration (v/wt). Siddiqui and Ahmed [19] asserted that pesticide induced stress in plants could trigger the formation of phenolic compounds, such as the isoflavones - genistein and diadzein, phenolic acids and hydroxycinnamic acid derivatives such as hydroxybenzoic acids, ferulic acids, p-coumaric acids, which are all potential inhibitors of germination and plant growth [20-22]. Perhaps some of these phytochemicals were triggered in response to chlorpyriphos and Ridomil at both 2% and 4% pollution concentrations.

Legumes are important in nitrogen fixation in soils and have increasingly become models/markers of toxic effects of chemicals (such as pesticides) in the environment, on plants. Ahemad and Khan [23] reported that fipronil (an insecticide) was stressful to legumes including chickpea, pea, lentil, and green gram, and observed the negative effects of the insecticide on shoot dry biomass, chlorophyll content, root nodule numbers and biomass. Chlorpyriphos had a significantly lower root nodule number/plant than Ridomil. Though an interesting observation on its own, the absence of a corresponding significance in root nodule biomass/plant makes it rather difficult to conclusively declare that chlorpyriphos affected root nodule formation more than Ridomil (see Table 3). We observed that control plants had significantly higher root nodule number/plant and root nodule biomass/plant, than plants in pesticides treated soils (Table 4). These attest to the negative influence of both pesticides on these legume parameters. According to Anderson *et al*., [24], pesticides could affect nodulation in legumes by; reducing carbohydrate supply to existing nodules; directly affecting rhizobial survival/activity in the soil; inhibiting biochemical signaling in plant roots that initiate/stimulate rhizobial attachment, inhibiting cell division of rhizobium, amongst others. Obviously, as described for use on cocoa, the pesticides had inhibitory effect on one or more of the above mentioned factors, in soybean.

The capacity of these pesticides as stressors, no doubt, owes much to their half lives in the environment. In this regard, while metalaxyl, the active ingredient in Ridomil has a half life of eight weeks (two months) and readily leaches in soil [25]; Sing *et al*., [26] asserted that chlorpyriphos has a half life of 36-46 days. Our findings, in soil exposed to natural illumination, suggesting that toxicity to soybean seed germination abates by the sixth week (as no significant differences were observed due to the pesticides concentrations in the sixth week after initial application), are not far-fetched. Glesy *et al*., [27] in a review on evidence for classification of chlorpyriphos and its metabolite - chlorpyriphos oxon as a: persistent organic pollutant (POP), and persistent bioaccumulative and toxic (PBT), concluded that there was not sufficient justification for the classification of chlorpyriphos as a POP or PBT. It appears our results on seed germination of soybean, add to, rather than subtract from their position; keeping in mind that manufacturer recommended preparations were used. On the other hand, root nodule formation, determined after three months of pesticides application, appeared a sensitive marker of chlorpyriphos and ridomil stresses, as nodule number and biomass were significantly reduced in the 2% and 4% treatment groups, as compared with the control.

Positive interaction between two pesticides should lead to a significantly higher response (by the marker), than is observed for equivalent single pesticides [15]. For both seed germination and root nodule formation, the effect (reduction) induced by a combination of both pesticides was not observed to be more than that due to chlorpyriphos or Ridomil alone. This implies a reduced likelihood of interaction between these two pesticides, for an enhanced stressful effect, when they are present together in the soil, on these legume parameters. One limitation of this study might be that the seeds were recovered after 72 hours for estimation of germination rate, rather than allowing them more time to perhaps germinate at their own pace. But a lag/delay in seed germination is itself a response to environmental stress [17].

## 5. Conclusion

This study investigated the effect of chlorpyriphos and Ridomil, singly and in combination on seed germination and root nodule formation in soybean, *Glycine max*. Pesticides were prepared in accordance with manufacturer recommendations for use on cocoa. More than Ridomil and Combination, significantly diminished percentage germination due to chlorpyriphos was observed at two, three, and four weeks after pesticides application. The control (0%) had significantly higher germination than 2% and 4% groups, up until the fifth week. Root nodule formation was also significantly reduced, due to pesticides stress at 2% and 4% (v/wt) pollution levels; as the control also had significantly higher root nodule number/plant, and root nodule biomass/plant than 2% and 4% groups. The chlorpyriphos and pesticide combination groups both had significantly lower root nodule number/plant, than Ridomil, but this was not reinforced by a corresponding difference in root nodule biomass/plant between the pesticide types. Pesticides application at 2% and 4% negatively affected germination and root nodule formation; but there was no indication of an enhanced effect due to pesticides combination, as chlorpyriphos negatively affected tested parameters more than the tested equivalents of Ridomil and their Combination.

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