Republic of Zambia

Ministry of Agriculture

Zambia Agriculture Research Institute

MICROBEBIO TECHNICAL REPORT

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Contents

Introduction

The world is faced with an increase in demand for more food to be produced exacerbated by a rapid rise in population. This not only adds pressure on the current production systems but threatens to comprise the ability of future generations to meet their daily food requirements (Tilman et al, 2002; Foley, 2005). Chemical fertilizers and pesticides have in the last 50 years contributed towards increasing food production across several landscapes, including Africa (Matson, 1997). However, due to logistical and budget constraints many developing countries (especially in Africa) have been unable to meet the required threshold of fertilizer and pesticide inputs, essential to drive their food production to the levels of the western world. This has resulted in yield declines, crop damage due to pests, soil nutrient mining (Maston, 1997), which is accelerating soil degradation to alarming levels. To mitigate this, agrarian systems are slowly tilting towards more sustainable and environmentally friendly means of production while reducing dependency on synthetic fertilizers and pesticides. The aim of this school of thought is to reduce chemical inputs without negatively affecting crop productivity, but to do this, a technology that can allow even resource poor farmers to produce more with less, without having adverse impact on the ecosystem and production system needs to be considered.

Biostimulants such as soil enhancers, conditioners, activators, have in the recent past generated a lot of interest within the scientific community because they have been deemed to significantly increase yield. These microbial amendments, which mostly comprise plant growth promoting rhizobacteria, plant hormones, fungi, and algae, and when applied in small quantities increase yield (Sharma et al., 2013), they elicit responses in plants resulting in better nutrient uptake form the soil. However, it must be noted that the bioproducts may need to be used in combination with other inputs such as conventional fertilizers and pesticides, for more efficient crop management.

It is from this background that the Zambia Agriculture Research Institute (ZARI) was requested by COMESA/ACTESA to undertake an evaluation of bioproducts microbebio soil vigor, microbebio aqua activator, and microbebio X1 nematicide, for the purpose of introducing them as amendments in Zambia. Glasshouse pot studies were established to test their (microbebio products) efficacy for nematode control and yield increase for major crops (horticulture, cereals, and other grains) commonly grown in Zambia. Three test crops namely maize, soybean, and tomato were used in 60 day trials. The major findings from these trials indicate

that the products are able to perform as claimed by the manufacturer, however, further studies will be conducted in the field by ZARI to validate the preliminary results.

This report intends to communicate to COMESA and other stakeholders on the implications of using the microbebio products for crop production in Zambia.

Objectives

The overall objective for setting these experiments was to evaluate the efficacy of four microbebio powder and granular based bioproducts, as soil amendments and control agents for soil borne pest and pathogens, and yield increase to use for crop production in Zambia.

The specific objectives are as outlined below:

1. To determine the effect of soil vigor application on the yield of pot grown soybean plants.
2. To evaluate the effect of super soil activator application a granular microbe fertilizer on biomass production in maize.
3. To determine the effect of aqua activator application on maize yield.
4. To evaluate the efficacy of using microbebio X1 bionematicide as a control agent for soil borne nematodes.

Materials and Methods

Pot grown plants were used to evaluate the efficacy of the four microbebio products (soil vigor, super soil activator, aqua activator, and X1 bionematicide), using glass and screen house facilities at Mount Makulu Central Research, Chilanga, Zambia. The four bioproducts which were supplied alongside Material Safety Data Sheets (MSDS) were and stored in a cool dry place away from direct sunlight prior to testing.

Three test crops namely soybean (soil vigor), maize (aqua activator and super soil activator), and tomato (X1 bionematicide) were used. A field soil obtained from Mount Makulu Research station was used in this study.

Aqua Activator Experiment

Trial set up and management

The trials for aqua activator were conducted between March and June 2017 inside and outside the glasshouse for 50 days. A commercial early maturing maize (*Zea mays*) variety SC403 obtained from SEEDCO, was pregerminated for 72 hrs in the dark at room temperature prior to planting. The maize seedlings were carefully transplanted into polythene pots containing 10kg field soil sieved to a particle size of 2mm, and irrigated to field capacity (figure 1).

Figure 1: *Left panel-***Pots field with field soil from Mount Makulu, arranged in a complete randomized design and watered to field capacity prior to planting the aqua activator trial.** *Right panel-***Pregerminated maize seedlings being given a final sprinkle of water before transplanting into pots**

Two experiments were set up for this trial; the first experiment had six treatments arranged in a complete randomised design (CRD) with five replications (see treatment description below), the second study comprised two treatments (aqua activator and full rate NPK fertilizer).

T1 – Control

T2 – Microbebio aqua activator seed dressing + half rate NPK (100Kg/ha)

T3 – Microbebio aqua activator seed dressing + full rate NPK (200Kg/ha)

T4 – Microbebio aqua activator soil dressing + half rate NPK (100Kg/ha)

T5 – Microbebio aqua activator soil dressing + half rate NPK (100Kg/ha)

T6 – Full rate NPK (200Kg/ha)

The microbebio aqua activator solution was prepared by dissolving 1g of product in 260mL deionised water (which is equivalent to the application rate for one hectare), and added as a 1mL soil drench for T4 and T5 (figure 2), while the seedlings for T2 and T3 were dunked into the concentrate and planted immediately. Likewise, for the second study, the solution was prepared as described above but was applied as a soil drench only.

Repeat applications of the soil drench were done 20 and 40 days after planting (DAP) to T2, T3, T4, and T5 in the first experiment, while in the second trial only the sole aqua activator treatment received the dose as described above. Fertilizer was applied at planting according to treatment, calculated based ono volume of soil contained in the pots.

All plants were kept well-watered by maintaining 80% evapotranspiration throughout the growing period up to harvest (50 DAP) by supplying 200mL of water every 2 days when the plants were small and 800mL as they grew larger. An incidence of fall army worm attack was observed in the early stages for both experiment, but was controlled with 5% EC cypermethrine, after which, there was no further occurrence.

Figure 2: *Left panel* ***- Aqua activator being applied as a soil drench to pot grown maize plants 20 days after planting.*** *Right panel* ***- Maize plants in the microbebio aqua activator experiment 23 days after planting.***

Measurements and data analysis

At harvest, plants heights were measured using a tape from the base of the plant just above the soil to the growing tip and data was carefully recorded according to treatment. After excising the shoot, roots were washed under a stream of water with care taken not to lose root mass; root lengths were recorded for individual plants as above. Fresh root and shoot plant material were oven dried at 80˚C in the oven until a consistent weight was obtained, prior to determination of root and shoot biomass production on dry weight basis. Analysis of variance to determine the effect of aqua activator on plant growth and biomass production for the maize across treatments was performed, at 95% confidence interval using Genstat statistical package. The data are graphically presented as means according to variables and treatments in the results section.

Super Soil Activator Experiment

Trial set up and management

In order to determine the effect of the super soil activator on biomass yield in maize, the same variety was used as above was established outside the glasshouse facilities at Mount Makulu. A CRD study involving three treatments (described below) with seven replications was set up for 40 days in 10 litre pots containing field soil from the research station, processed as described above.

Treatment 1 - super soil activator sole

Treatment 2 - super soil activator + half rate NPK fertilizer (100Kg/ha)

Treatment 3 - full rate NPK fertilizer (200Kg/ha)

Fertilization and plant management was done in a similar manner as described in the aqua activator experiment.

Measurements and data analysis

Forty days after planting, the shoot and root biomass, plant heights, and root lengths were measured (see description above). Analysis of variance with Tukey's post hoc HSD test for mean separation was performed at 95% confidence interval, to assess treatment performance and are presented in the results section.

Soil Vigor Experiment

Trial set up and management

To evaluate the performance of soil vigor, Lukanga a non-self nodulating soybean variety obtained from ZARI was grown in 5 litre polythene pots (planted on the same day as the maize) containing 5kg field soil and processed as described above. Seeds were selected for uniformity (size) and pre-germinated for 48 hrs in the dark at room temperature. The seedlings were then transplanted into pots irrigated to field capacity prior to planting and arranged in a CRD with five replications, according to treatments described below:

Treatment 1 – Control

Treatment 2 – Rhizobium + full rate NPK

Treatment 3 – Soil vigor sole

Treatment 4 – Soil vigor + full rate NPK (200Kg/ha)

Treatment 5 – Soil vigor + half rate NPK (100Kg/ha)

All plants were grown under well-watered conditions (up to harvest) following the respective treatments. For Treatment 2, soybean inoculant containing a strain of *bradyrhizobium japonicum* (CAIT 102), was applied as a seed coating with fertilizer at the rate of 200Kg/ha supplied at planting respectively. The soil vigor was applied to treatments 3, 4, and 5 by firstly diluting 2g in 10mL deionised water, to make a concentrate, after which, the solution was

further diluted with 90mL DI water to make up to a volume of 100mL. The microbebio treatments were applied by adding 1mL of the solution as per treatment (figure 3), and were immediately followed by irrigation to field capacity (as described in the microbebio soil vigor information sheet). Full rate and half rate fertilizer treatments were applied as describe above to treatment 4 and 5 respectively.

The microbebio treatments were repeated 3 times at 20, 30 and 40 days after planting.

Figure 3: *Left panel* ***- Soil vigor and aqua activator solutions before application.*** *Right panel* ***– soybean plants arranged in a CRD according to treatments in the glasshouse.***

Measurements and data analysis

Forty days after planting, shoot and root biomass were measured following the procedure described above. Analysis of variance with Tukey's post hoc HSD test for mean separation was performed at 95% confidence interval to assess treatment performance and are equally presented in the results section.

Microbebio Bionematicide Experiment

Trial set up and management

Black soil was collected from rice fields at Mount Makulu and was left to dry for 48 hours, then the soil was sterilized for 30 minutes at 121°C. The cooled sterilized soil was transferred into 5Kg polythene pots and taken to the screenhouse in readiness for planting. Three tomato seedlings, variety tengeru, obtained from a nursery at the research station were transplanted at 15 cm spacing in a triangular pattern at a depth of 1cm into each pot. The plants were then arranged in a CRD comprising the following treatments:

Trt1 Control: No Treatment + Inoculation of 60 Nematodes

Trt2 Conventional Bionematicide + Inoculation of approx. 60 nematodes

Trt3 Conventional Bionematicide + Inoculation of approx. 40 nematodes

Trt4 Conventional Bionematicide + Inoculation of approx. 20 nematodes

Trt5 Bionematicide + Inoculation of approx. 60 nematodes

Trt6 Bionematicide + Inoculation of approx.40 nematodes

Trt7 Bionematicide + Inoculation of approx. 20 nematodes

Nematodes were isolated from soil samples collected from a tomato farm in Chilanga (in close proximity to the research station) by sampling the top soil (0-20 cm). Samples were immediately placed in clean plastic bags and transported to Mount Makulu Entomology Laboratory for further processing. A modified Baerman funnel (extracting tray, wire mesh, filter paper and paper towels) was setup and used to extract the nematodes from the mixture of soil and macerated plant roots. A supernatant (water suspension) was collected and transferred into clean glass jars and left to stand for 30 minutes. Some of the remaining water was decanted and carefully transferred onto petri dishes for examination under a light microscope for the presence of nematodes (figure 4).

Figure 4: Nematodes being observed under a microscope.

The number of nematodes was established by using a counting dish and then transferred with a sterile syringe to the pots in order to achieve the following inoculation numbers per pot: 60, 40, 20, and control (no nematodes) nematodes, replicated 3 times. Basal fertilizer was applied to each pot at the recommended rate for tomato production in Zambia (figure 5).

Figure 5: Tomato plants arranged according to treatments in the greenhouse

A commercial nematicide was used alongside the X1 microbebio bionematicide applied as a soil amendment 30 days after the potted plants had been inoculated with nematodes, following the recommendations contained in the product information sheet.

Measurements and data analysis

Seven days after application of treatments, soil samples were collected from each pot to determine the number of surviving nematodes. The growth of plants was also monitored throughout the period paying particular attention to visual symptoms of nematode attack. Sixty days after transplanting, shoot and root biomass was determined by drying the plants in the oven at 80˚C for 72 hours. The samples were immediately measures using a laboratory balance, data was analysed using Genstat statistical package with individual treatment means separated by Tukey's HSD test at 95% confidence interval.

Results and Discussion

Performance of soil vigor in soybean

The data on shoot and root biomass were analysed in a CRD to test statistically the differences among treatments combinations for soil vigor in soybean. Analysis of variance indicated that the differences between soil vigor and the recommended rhizobium with fertilizer treatment were not statistically significant for both shoot and root biomass (figure 6). However, addition

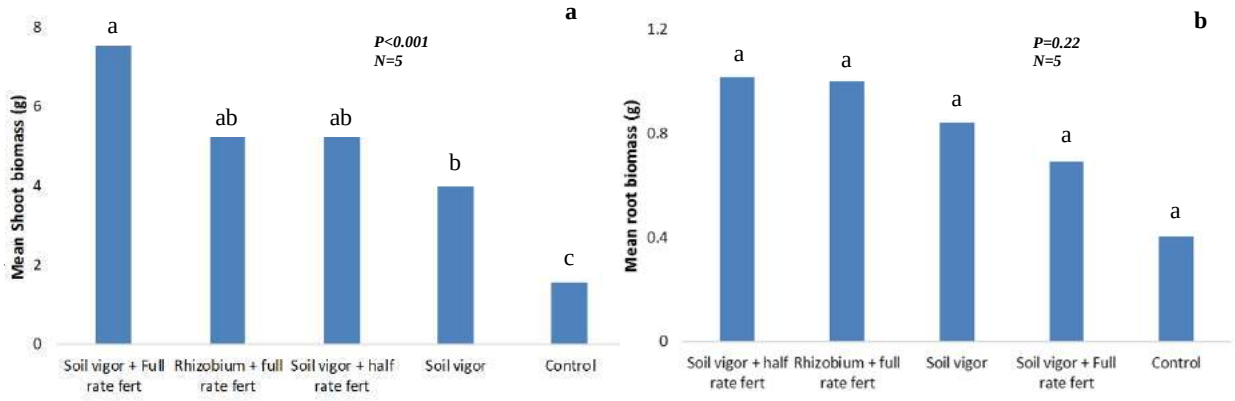

Figure 6: (a) - Mean shoot biomass for soybean plants treated with soil vigor 0, 20, and 30 DAP; (b) - Root biomass for different soil vigor, fertilizer , and rhizobium combinations. Data are means of 5 plants harvested 40 DAP, analysis of variance was performed, with Tukey's HSD test. Different letters indicate statistical differences at 95% confidence interval.

of soil vigor in combination with full rate fertilizer significantly increased biomass by 30%, when compared to the rhizobium + fertilizer application (current recommended practice for soybean production in Zambia). The results further suggest that soil vigor when applied as a sole application equally increased biomass two-fold, when compared to the control. This implies that the plants showed significant responses to soil vigor application thereby resulting in more biomass production, which is a proxy of grain yield and plant performance as influenced by availability of adequate nutrition. Despite not showing any statistical differences in root biomass, application of soil vigor considerably increased root dry matter production by 50% over the control. It can be argued that healthy root system results in more competitive plants that are capable of absorbing additional nutrients and water from the rhizosphere (Reynolds and Thornley,1982; Bolinder et al. 1997).

A further analysis of shoot: root ratio data revealed that the soil vigor + full rate fertilizer increased shoot growth over the roots by 60%, possibly implying that the plants were exponentially responding to plant nutrient availability and uptake and perharps allocating more

carbon towards above ground yield components (figure 7). This scenario might subsequently result in higher grain yields because the plants would be transpiring and photosynthesizing more, while having access to more light and nutrients. Soil vigor is an amendment that contains microbes, which are able to enhance plant nutrient uptake within the rhizosphere (http://www.microbebio.com/product/microbebio-nature-vigor).

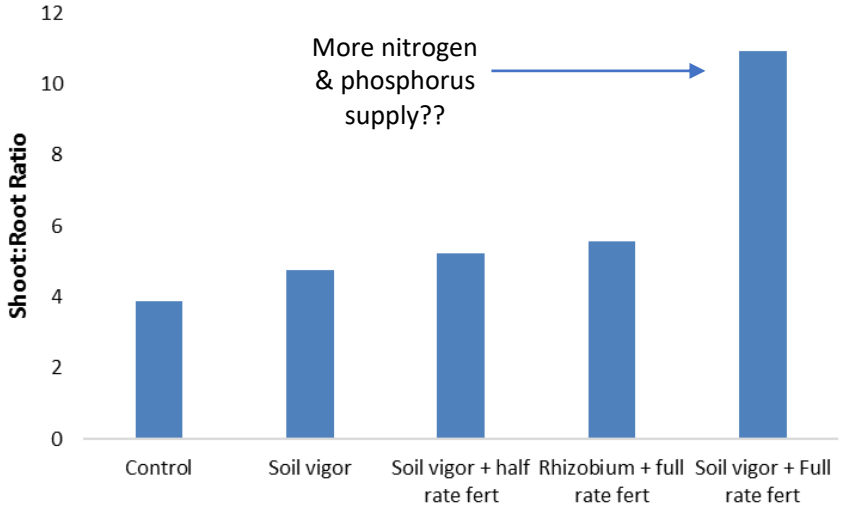

Figure 7: Shoot: root ratios for soybean plants treated with soil vigor, rhizobium, with and without fertilizer supplied at half and full rate were applicable. Data are means of 5 plants harvested 40 DAP.

Therefore, the results obtained from this study are consistent with the assertion by microbebio; for instance, a study involving *pisum sativum* and *phaseolus valguris* grown under varying nitrogen supply regimes, observed a strong positive correlation between increased shoot: root ratio and biomass production or yield (Andrews et al. 1999). For that reason, it is highly probable that soil vigor contributed to plant nutrient uptake within the rhizosphere for the soybean plants grown in pots (figure 8).

Figure 8: Rhizosphere showing the root architecture of a soybean plant taken from the pot study 40 days after planting.

Performance of Aqua Activator in maize

Analysis of variance performed on plant heights and root lengths data to test the effect of aqua activator on maize. The data did not reveal any statistical differences ($P>0.05$) between the means of plants treated with aqua activator and full rate fertilizer, for both variables (figure 9 a & b). A similar pattern was observed for shoot and root biomass yield, though the aqua activator treated plants outperformed the ones supplied with full rate NPK fertilizer (Figure 9 c & d). The plants did not show signs of nutrient deficiency throughout the experiment. It must be pointed out however, that control plants (data not shown) exhibited signs of severe phosphorus deficiency concomitantly with stunted growth when compared to the aqua activator and fertilizer treated plants. Conversely, a combination of fertilizer and aqua activator exhibited a very high plant growth vigor phenotype between day 10 and 40 (data not shown).

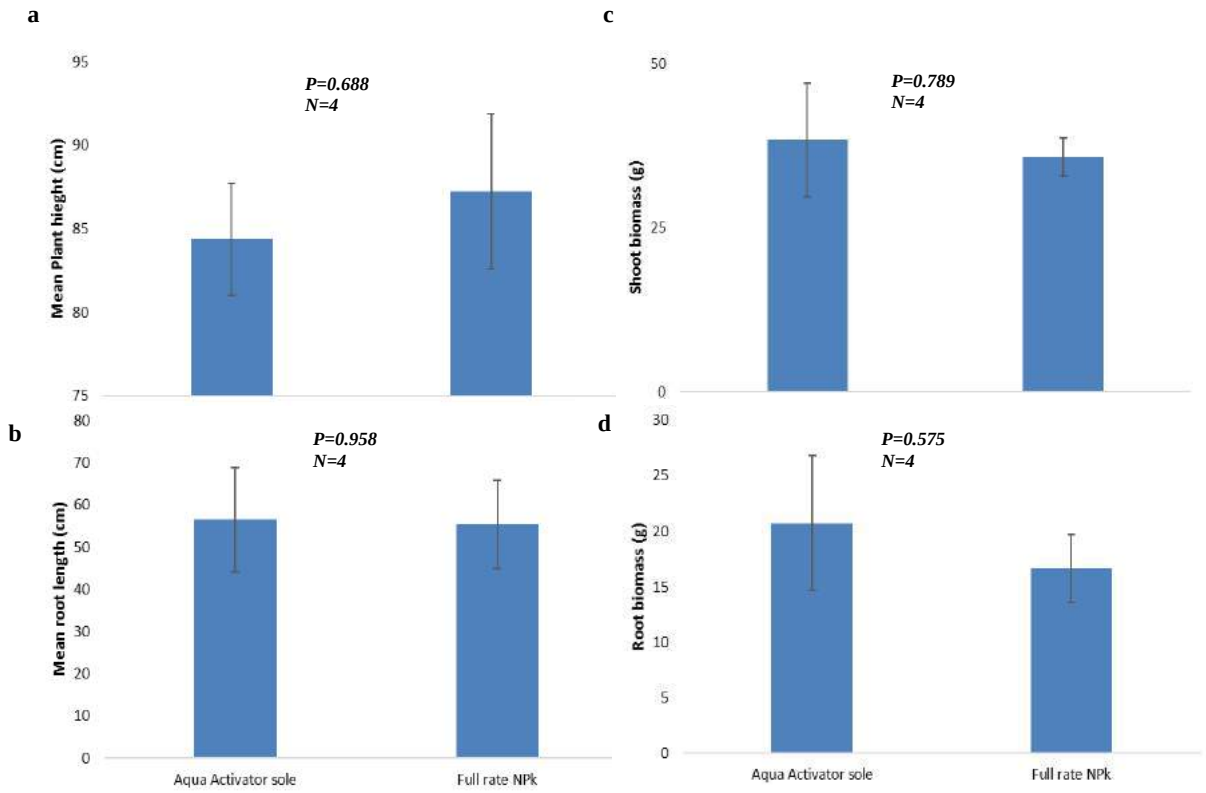

Figure 9: (a) – Plant heights for maize plants supplied with aqua activator and fertilizer; (b)- root length for the same maize plants as in (a); (c) shoot biomass; (d)root biomass. The aqua activator was applied as soil drench 0, 20, and 40 DAP. Data are means of4 plants harvested 50 DAP, analysis of variance was performed to test the treatment effects at 95% confidence interval.

However, caution must be applied when interpreting these results because of the limited growth period by which the plants were subjected to, before being harvested. It would be interesting to observe how the aqua activator would perform under full scale field trials covering the entire growing period of maize. It must be emphasised here that there is a debate in the scientific community involving plant microbe-interactions, biostimulants, microbial soil amendments containing enhancers, conditioners etc., as to whether these can replace chemical fertilizers, with a number of scientist recommending a reduced fertilizer rate application over a sole application of the bioproduct. The latter view is particularly important to avoid excessive soil nutrient mining (if growing heavy feeder crops), which would then result in a negative effect on soil health and nutrient supply in the long term. It is undeniable that the aqua activator in this case demonstrated superior performance over the chemical fertilizer. As to whether this performance can be sustained is a question begging to be answered in subsequent trials.

Figure 10: Control plant showing signs of phosphorus deficiency, highlighted by red arrow. Adjacent plants were supplied with combinations of aqua activator with full and half rate NPK fertilizer respectively. Picture was taken 23 days after planting following the second aqua activator treatment.

Performance of super soil activator in maize

The performance of super soil activator was tested by analysing data for plant heights and shoot biomass collected 40 days after planting. Analysis of variance ($P=0.699$ and $P=0.88$) indicated no statistical differences among treatment means for plant heights and shoot dry matter respectively (figure 11 a and b). These results suggest that plant responses to super soil activator were positive. Interestingly, when shoot biomass is considered, combining soil activator with half rate NPK seemed to have slightly outperformed the full rate NPK treatment, though not statistically significant. This may require further investigation to establish the mechanisms by which plant nutrients are made available in the rhizosphere resulting from interaction between plants and microbes contained in soil activators, when supplied with sub-optimal chemical fertilizers.

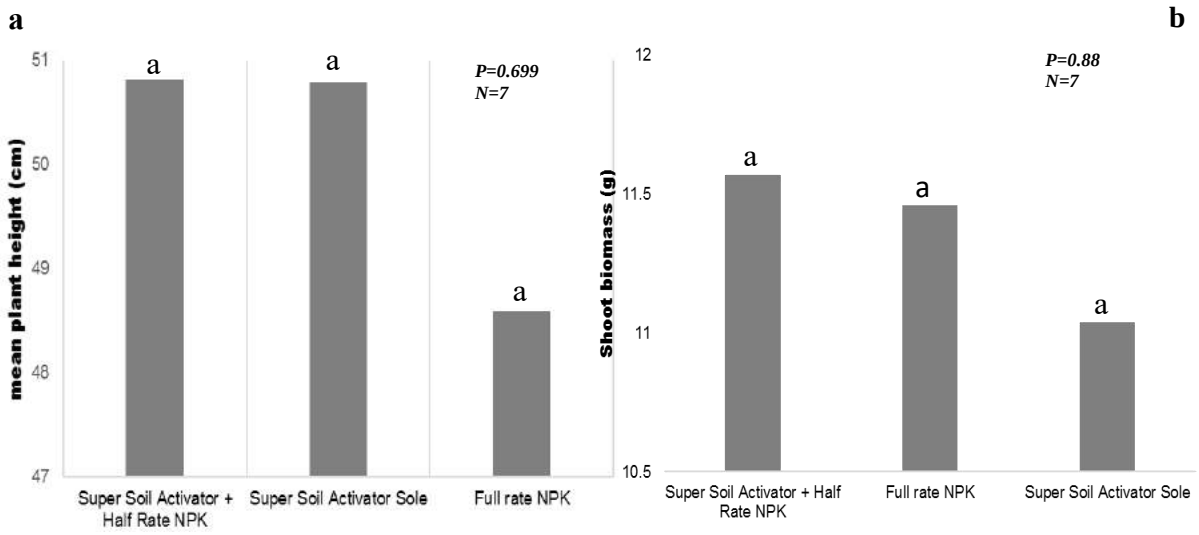

Figure 11: (a) – Plant heights for maize plants supplied with super soil activator and fertilizer; (b)- Shoot biomass for the same maize plants treated as in (a); The soil activator granules were applied at planting as described in the methods. Data are means of 7 plants harvested 40 DAP; analysis of variance was performed to test the treatment effects followed by Tukey's HSD test at 95% confidence interval. Similar letters indicate no significant differences across treatments.

Further assessment of the roots was done to confirm whether soil activator contributed to below ground biomass accumulation. Interestingly, root length and root biomass data revealed similar trends in terms of performance to what was observed in the shoots (figure 12 a and b). Furthermore, there was a positive correlation (r^2=0.79) between root growth and shoot biomass accumulation. Therefore, for plants to be more productive (more biomass per input), as earlier argued, the root system needs to be well established for better nutrient and water uptake for the plant to be able to support above ground development (Wang et al., 2005; Mantelin, 2003). This can consequently lead to better biomass partitioning into essential yield increasing components (leaves and reproductive parts) in the plant. However, due to time constraints and the complexity involving mechanisms by which biostimulants such as soil activator contribute to nutrient uptake in plants, this study did not determine nutrient use efficiency (NUE), a more accurate estimate of productivity. Future studies should investigate this relationship to gain better understanding of what is happing within the crop production system.

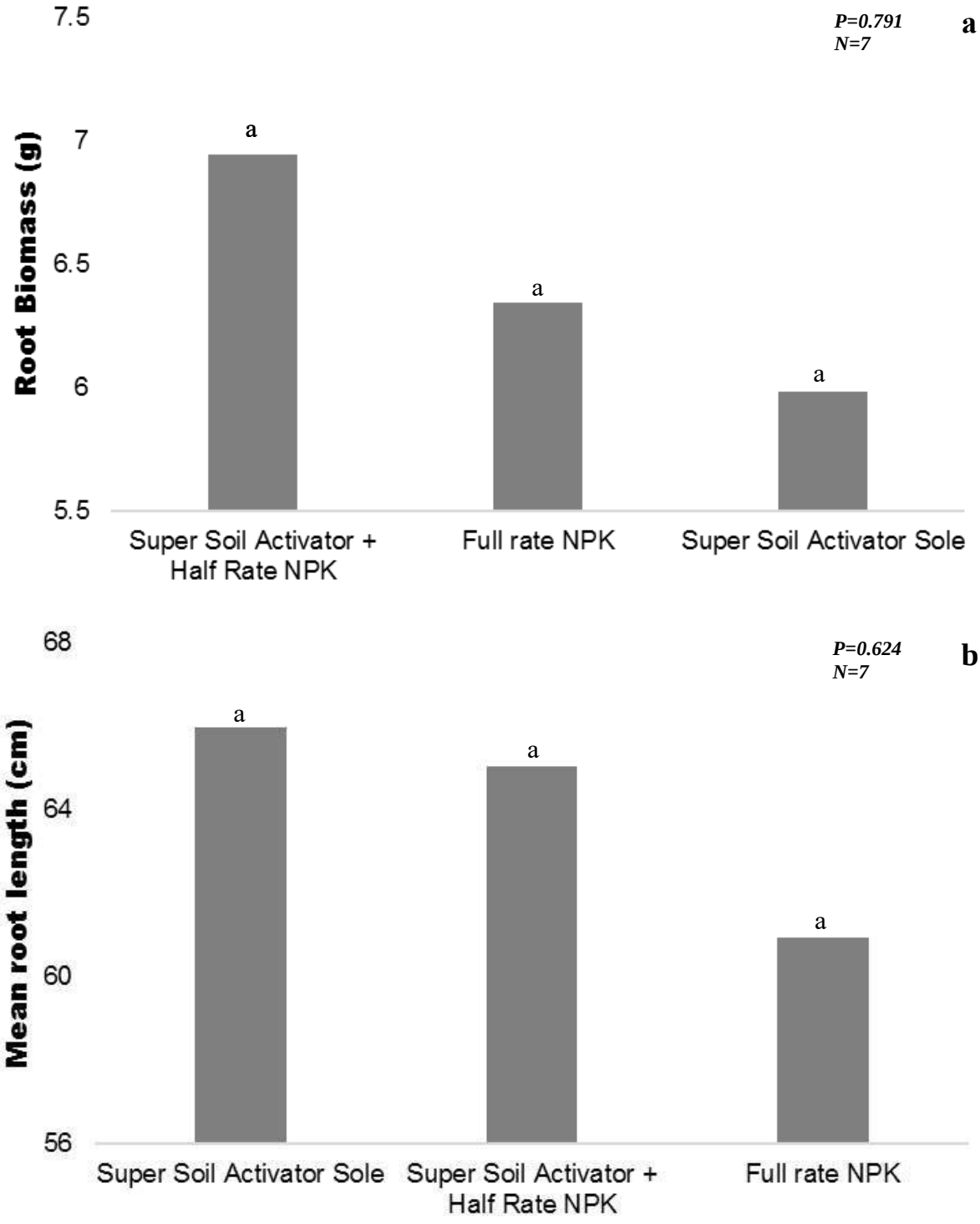

Figure 12: (a) – Root biomass for maize plants supplied with super soil activator and fertilizer; (b)- Root lengths for the same maize plants treated as in (a); The soil activator granules were applied at planting as described in the methods. Data are means of 7 plants harvested 40 DAP; analysis of variance was performed to test the treatment effects followed by Tukey's HSD test at 95% confidence interval. Similar letters indicate no significant differences across treatments.

Performance of X1 bionematicide in tomato

To determine the efficacy of X1 bionematicide, its performance was evaluated against a commercially available synthetic control agent applied to the same set of tomato plants as described in the materials and methods. Soil samples collected from the inoculated pots (with nematodes, see methods) and observed under a microscope indicated inactive nematodes in both the commercial and bionematicide treated soils. This result seems to suggest that the nematodes were merely immobilized but did not die, possibly because the application of the bionematicide was only done once. It is worth noting that when plant roots were observed for gal formation, there was no evidence of attack or infestation. Further follow up investigations were then carried out in the laboratory, by plating soil samples on petri dishes and treating them with a normal, double, and triple dosage of X1 bionematicide respectively (applied as a solution to each petri dish). When observed under a microscope, the normal dosage did not seem to have an effect on the nematodes, but as the concentration doubled and tripled the parasites were either immobile or appeared to be dead. We therefore postulate that as the concentration of the fungal spores (contained in the bionematicide) increased, the nematodes were unable to maneuver through the growing mesh of growing mycelial fungi to penetrate the root tips, thus leaving the crop healthy.

Studies have likewise demonstrated that beneficial fungi and other biological control agents can induce defense responses in plants, compete for nutrients (with the pathogen), and exude compounds which can immobilize the pathogen in the rhizosphere, thereby limiting attack to the crop (Schouteden et al., 2015; Berruti et al., 2015; Igiehon and Babalola, 2017). However, it has been established that the timing of application is important to enable the beneficial microorganism to establish adequately in the soil for effective control to occur (Berruti et al., 2015). Our study observed the soils at 14 and 30 days after application of bionematicide, which may not have been sufficient for the active ingredients (microorganisms) work. We recommend that further studies involving X1 bionematicide be conducted in-situ, on fields with a known history of nematode infestation for a longer period. This would take some considerable time for the fungi spores to build up and start parasitizing the nematode pests whose life cycle is approximately 28-30 days in most species.

In general, plant growth was normal without displaying any symptoms of wilting and drooping of leaves caused by nematodes (figure 13). This can be correlated by the shoot and root biomass

data, which did not exhibit signs of nematodes having an impact on the growth of plants (figure 14 a and b).

Figure 13: Tomato plant showing no visible signs of nematode attack. picture was taken 40 days after planting

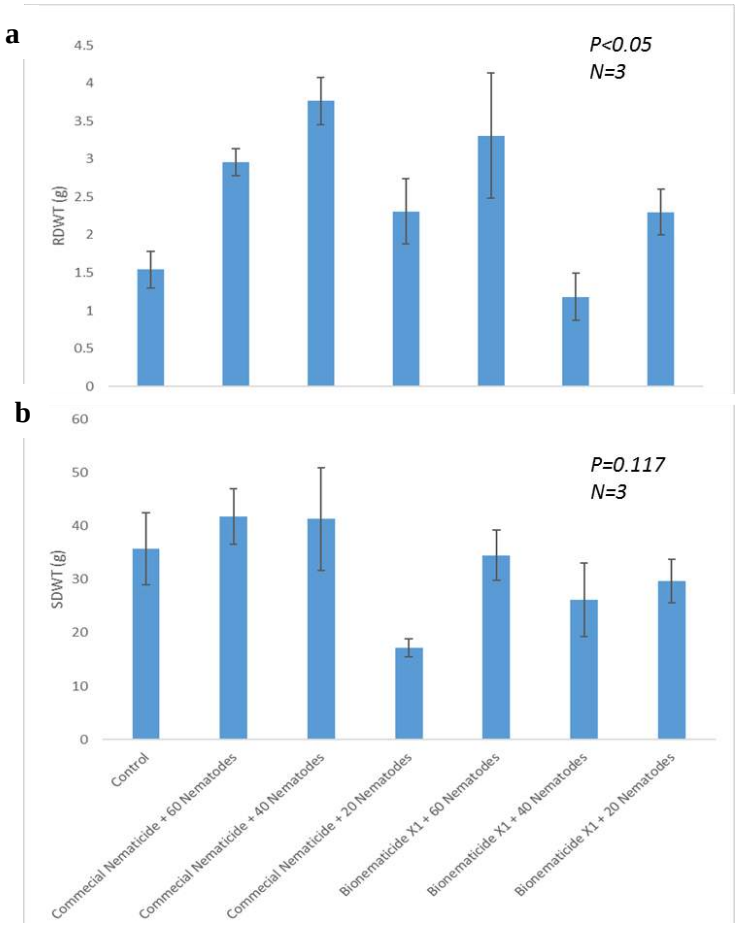

Figure 14: (a)-Root biomass for plants treated with X1 bionematicide and a synthetic nematicide, pot were inoculated with 20, 40, and 60 nematodes; (b)- Shoot weights for tomato plants treated as in (a); Data are means of 3 plants harvested 60 DAP; analysis of variance was performed to test the treatment effects.

Conclusion

This study set out to evaluate the efficacy of four microbebio powder and granular based products as soil amendments and control agents for soil borne pathogens. The research has also shown that super soil activator, soil vigor and aqua activator significantly increased plant biomass (root and shoot) when compared to the controls. Taken together, these results suggest that the three soil enhancers can perform in the field, thus putting more grain in the farmer's hands through efficient nutrient uptake and better agronomic efficiency (though not the scope of this study); subsequently making them producing more with less input. However, there is need to understand the long term impact on soils (organic pool, inherent soil fertility status), it would therefore be of paramount importance in the short term to combine the use of the product with a reduced fertilizer application rate for maximum benefits.

The second major finding was that the bionematicide X1 can be applied in fields were infestations by nematodes are relatively low as a control agent. As the population of the pathogen increase, the number of spores (from the product) would be augmented correspondingly with time. This governed by the right dosage, timing and frequency of application, may provide a novel product for the control of nematodes while at the same time contributing to increased yields. Lastly, this research provides a framework for the exploration of further research in fields with a history of nematodes as well as mechanisms, which control the mode of action. Bionematicides such as X1 when used as part of a complete pest management program can reduce crop damage by plant-parasitic nematodes.

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