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Effects of Nitrogen and Sulphur On *Brassica Juncea* (Cv. Caliente 199) Used for the Biofumigation of Potato Cyst Nematodes (*Globodera pallida* (L.))

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Abstract –:*Glasshouse experiment was setup to find out the suitable ratio of Sulphur and Nitrogen for optimal Biofumigation for suppression of Potato Cyst Nematode (Globodera pallida [L.]) using Brassica juncea (Cv. Caliente 199) and to compare the efficacies of Meldola's blue stain and Trehalose-based methods of quantification for assessing egg viability of potato cyst nematode (PCN). sixteen treatment combinations were used and the parameter assessed was percentage PCN egg viability.*
statistical analysis was conducted using 'R' software. results of two-way ANOVA showed significant difference in the treatments on PCN egg viability (p = 0.005). Regression analysis conducted using "R" software to compare Meldola blue stain and Trehalose methods revealed slight correlation (r = 0.3) between the two methods of PCN eggs viability test. hence, both the null hypotheses were rejected.
Therefore, it is recommended that treatment ratio of 50kg/ha N and 60kg/ha S should be used forbiofumigation in the UK, due to its positive effects in suppressing PCN egg viability. similarly, Trehalose method is recommended as substitute for Meldola blue stain in PCN egg viability test because the two are positively correlated but the former is faster and less tedious.

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I. Introduction

Potato Cyst Nematode (PCN) poses a big challenge to potato producers in the UK. it is responsible for annual loss of 9% of UK total production (Moxnes, 2007). according to a recent survey, PCN alone costs the potato growers in the UK up to £25.9 million annually (AHDB, 2016).Studies have proven wide distributions of *Globodera pallida* (responsible for the problem) in the UK. In his study on distribution and occurrence of PCN on the potato cultivated lands of Wales and England, Minnis,*et al. (*2002) showed that 62% of the infested lands had a density of about 10 eggs g^{-1} soil. Ngala,*et al.* (2014) stated that about 64% of fields subjected to cultivation of potato in England and Wales were PCN infected, and 92% of these infections were associated with *G. pallida.* conversely, in a recent unpublished survey by Dybal,*et al.*(undated) the percentage of lands infested by PCN was reported to have dropped to 48%.

"Biofumigation is the agronomic practice of using volatile chemicals (allelochemicals) released from damaged tissues of brassica plants to suppress soil-borne pathogens, weeds and pests", (Mattner,*et al.* 2008). In the UK, brassica plants used as biofumigants have shown efficiency in the suppression of population of PCN. phytochemicals such as glucosinolates (GSLs) were found to be released by Indian mustard (*Brassica juncea*) which undergo hydrolysis in the presence of enzyme myrosinase and change it to Isothiocyanate (ITC) which suppresses the nematodes (Ngala,*et al.* 2014).

Pest and pathogen suppression can also occur due to changes taking place in the rhizosphere of the biofumigant prior to its soil incorporation. The presence of other microbes that can produce myrosinase in the rhizosphere of biofumigant, such as *Aspergillus spp,* is found to enhance the biofumigation efficacy, which is termed as partial biofumigation (Ngala,*et al.* 2014). A glasshouse potted experiment conducted by Ngala,*et al.* (2015) revealed that prior to brassica incorporation, significant (p=0.027) increases in *G. pallida* egg mortality was observed for unsterilized soil compared to sterilized soil planted with brassica plants. Similarly, there was significant (p<0.001) increase in total microbial activities in the rhizosphere prior to incorporation in unsterilized soil compared to sterilized soil treatments. This strengthens the proposition of Motisi,*et al.*(2012), that the ITC released by the root in the rhizosphere of biofumigant crop might exert some influence on the existing microbes thereby changing the structure of the microbial communities, which indirectly changes the pathogens competitiveness or increases their antagonists' population.

Research indicates that glucosinolates are a sink for nutrients such as nitrogen and sulphur. These elements form part of protein synthesis and biofumigation is essentially an enzymic process that requires these elements for the synthesis of the final metabolites. Nikiforova, *etal.*(2003) reported that GSL was among the main sulphur containing compounds with 6% of the total sulphur in brassica contained in it. However, for opti-

mum GSL accumulation and consequent effective biofumigation to be achieved a proper combining ratio of nitrogen and sulphur need to be established. this is because alteration of one element in the system (process) prevents the other pathway. Van der kooij,*et al*. (2008) observed a significant difference (<0.05) in the interactions between N and S in the dry matter of *Arabidopsis thaliana* shoots. Martinez-Ballesta,*et al*.(2013) reported that low nitrogen supply along with high sulphur fertilization produced an increased GSL concentration in broccoli. Furthermore, excess nitrogen supply generally lowers (decreases) total GSLs. Similarly, any change in the proportion of nitrogen and sulphur in brassica tissue is accompanied by a corresponding change in the type or components of the GSLS produced. Zhao,*et al*.(1994) reported that increase in nitrogen rate in oilseed rape relatively increased the proportion of 2-hydroxybut-3-enyl against pent-4-enyl. Similarly, sulphur application produced greater effects if it is present in alkenyl group as compared to when it is a constituent of indole group.

Although biofumigation leads to suppression of PCN population, little seem to be understood regarding the agronomy of brassica (biofumigant) in relation to the ratio of sulphur and nitrogen for optimum control of PCN population. it is also crucial to compare the methods used in cyst viability test to come up with the simpler and more reliable assays. Therefore, this study will try to address these gaps.

OBJECTIVES –

- To determine the optimal proportions of sulphur and nitrogen inputs for *B. juncea* that readily reduce viability of the encysted eggs of *Globodera*spp. through biofumigation.
- To compare the Meldola blue stain with Trehalose methods of PCN viability assessment.

NULL HYPOTHESES–

- Nitrogen and sulphur proportion has no effect on egg viability or hatchability in biofumigation.
- There will be no correlation between Meldola blue stain and Trehalose assays in determining PCN eggs viability.

II. Methodology

Soil was collected from PCN-infected field near Crudgington, Shropshire in September 2016. About 2 - 2.5kg of the infected soil was placed and tied in 32 cotton bags and stored in oven to dry in the elutriation section of the nematology lab. Mass extraction of the cyst was carried out using Fenwick can. Batches of extracted cysts were dried at 15˚c in readiness for the experiment set-up.Cysts were counted under a stereomicroscope (x30), using a bespoke channelled aluminium slide and a pair of forceps. Forty cysts were counted and put into Eppendorf tubes, capped and kept ready for sealing in the nylon sachets. Eighty nylon sachets (about 3 x 4cm size) were prepared and 40 cysts were put in each and sealed, using a heat-sealer machine.

Sandy-loam soil was collected from field no. 52.772490 at Four Gates of Harper Adams University campus (UK) located on latitude $52^{\circ}\ 46^{/}$ n, longitude $2^{\circ}\ 25^{/}$ w (Google map, 2017). The soil was mixed with John Innes no. 2 compost in the ratio 5:1 and well homogenised using mixer machine in the glasshouse. the compost was used to reduce slumping within the pots. The soil $_{\mathbf{pH}}$ was 6.3 and the nitrogen/sulphur status of the soil was determined prior to mixing and pot-stocking of the soil using macro-kjeldahl/turbidometric methods and found to be 0.35/0.025% respectively. Automated irrigation facility was used. Ammonium nitrate (nitram®) (NH_{4NO3}) and elemental sulphur were used as treatments in varying quantities. Approximately 8kg of the mixed soil was introduced into each 25cm (10 inches) pot. The top 5cm soil was thoroughly mixed with the nitrogen/sulphur treatments (except the control pots). The 16 treatments were randomized and arranged in five blocks on the sliding bench in the glasshouse. The set-up comprised of 80 pots (five of which acted as control with no treatment given) properly arranged to receive equal illumination from the overhead light source and labelled accordingly.

Indian mustard (caliente 199) or *Brassica juncea* was sown at the rate of 17 seeds $pot^{-1}$and later thinned to 8 seedlings per pot (evenly spaced) to ensure proper rooting and seedlings establishment. the experiment was set under controlled glasshouse conditions of day and night temperature at 15 and 5ºC respectively, 60% relative humidity and 16hour photoperiod. The seedlings developed quickly and produced flower buds by the end of the 4^{th} week after sowing (WAS). At 5 WAS, most of the plants attained 35% florets. At the end of 6^{th} week the plants were carefully uprooted from the pots. The fresh biomass was carefully macerated using garden shredder and the shredded materials mixed with the soil and incorporated for 30s. The mixture was poured back into the pot with the sachet buried 10cmdeep. A litre of water was poured into each pot to induce hydrolysis. Pots were placed in their original place on the bench and left for aperiod of 3 weeks.

Viability Test– At the end of eighth WAS the sachets were dug out of the pots and taken to laboratory for viability test. The cysts from each sachet were divided and used for the two viability tests separately, visa-vis; Meldola's blue-stain and Trehalose-based quantification methods.

Meldola's Blue-Staining Bioassays–Twenty cysts from each sachet were put into distilled water and allowed to stay for seven days. Each sample was treated with 1ml portion of 0.05% (w/v) Meldola's blue staining solution and kept for 7 days. Aluminium block and a glass slide were used to gently crushed the stained cysts.

The crushed cysts were rinsed down into 100ml measuring cylinder and mixed well with 50ml of deionised water to form suspension. 1ml aliquot of theeggs suspension was pipetted onto a gridded glass slide. A 40x binocular microscope was used to count the number of viable and non-viable eggs. Non-viable eggs were capable of absorbing and retaining the blue stain, while viable eggs were unable to absorb the blue stain. Number and percentage of viable eggs $cyst^{-1}$were estimated using equations 1 and 2 respectively:

$$E = W \times \frac{e}{Ce} \qquad (1)$$

Where E = Number of Viable Eggs $Cyst^{-1}$, W = Quantity of Water (Ml) In The Egg Suspension, E = Number ⁄ Where %V is the Percentage Viability, *V Is* Number of Viable Eggs, N is the Total Number Eggs.

Trehalose Method of PCN Viability Test– A modified form of methodology used by Ebrahimi *et al*., (2015) was used for this test. Twenty cysts were placed into each of the eighty (1.5 ml Eppendorf) tubes and labelled appropriately. 200 µl of deionised water was added to each tube and tightly capped. The contents were warmed at 99ºC for 30 minutes in a thermomixer incubator (Eppendorf, Hamburg, Germany). The cysts were crushed in the tubes for 30s, using plastic pestles. The suspensions were spin and kept settling. 100 µlof the clear supernatants and a blank were placed in 80 portions of a well plate. 152 µlof master mix (mm) was added to each well and the blank. The well plate was slotted into spectrophotometer and set to read the samples and blank absorbance at λ = 340nm (raw data 1). The plate was taken out and Trehalase (enzyme) was added to each well plus the blank and allowed to react for 5 minutes. The plate was slotted again into spectrophotometer to record the second absorbance (raw data 2). Difference in the two readings was calculated using the simple arithmetic in equation 2:

FINAL DATA = (RAW DATA 2 – RAW DATA 1) – (AVERAGE BLANK 2 – AVERAGE BLANK 1) (2)

The parameters assessed include percentage PCN eggs viability using Meldola's blue stain as well as Trehalose methods. Two-way ANOVA was used to analyse the effect of treatments on the viability of PCN eggs, using 'r' statistical package. Tukey's multiple range test and Honestly Significant Difference (HSD) were also conducted to determine the variations between individual treatments. Regression analysis was also conducted using "R" to determine the type of correlation existing between the two methods of PCN viability assays visa viz, Meldola blue staining and Trehalose methods.

III. Results and Discussions

Treatment 14 (50 kg/ha N, 60 kg/ha S) produced least PCN viability of about 40%(figure 1). This agrees with the earlier research conducted by Rajab, *et al*. (2017) who similarly observed that highest GSL concentration (17.05 $\mu molg^{-1}$)was produced at that treatment combination. This is because Ngala,*et al*. (2014) observed that the mortality rate of *G. pallida* was linearly related to the concentration of GSLs released in the medium. Moreover, Martinez-Ballesta,*et al*. (2013) reported that low N supply together with high S fertilization produced an increased concentration of GSLs in broccoli. Treatment 15 produced highest percentage (%) viability of over 60% (figure 1). This could be due to very high proportion of N in the treatment (100 kg/ha N, 60 kg/ha S) which leads to low production of GSL and consequently resulted in low PCN mortality. This observation is strengthened on one hand by the work of Martinez-Ballesta,*et al*. (2013), as explained above,and on the other hand by the significant interactions of N and S (p= 0.005) as seen in table 1. The table also indicates that the *p*-values for both sulphur and nitrogen show significant differences for viable eggs per cyst and % viability respectively. Moreover, there were significant interactions between nitrogen and sulphur (p = 0.081 and p = 0.005) for viable eggs per cyst and % viability respectively. However, the interaction effect is not very strong (p = 0.08) in the case of viability of eggs per cyst. Tukey's multiple range test was conducted to findout the differences among the treatments and figure 1, indicated that treatments with letters "a", "ab" and "b" show significant difference among themselves. however, treatments with the same letters show no difference among themselves.

The results in table 1 and figure 2 show that there is slight correlation (0.3) between Meldola blue stain and Trehalose methods of PCN viability testat F = 9.92 and p = 0.002. This is clearly shown by the slight convergence of the dotted plots along the regression line. This alsoconcurred with the work of Ebrahimi,*et al*. (2015) who reported PCN viabilities of 88 and 86.1% for visual and Trehalose assessments respectively (at F = 6.24, df = 2, p = 0.001).However, Ebrahimi,*et al*. (2015) opined that there were elements of subjectivity in Meldola blue stain method of PCN viability assessment.

IV. Conclusion

The results of the experiment indicate that both the null hypotheses would be rejected because treatment 14 with nitrogen-sulphur ratio 50 kg/ha-60 kg/ha respectively shows the lowest % PCN mortality. Hence this could be the recommended ratio in biofumigation for optimum PCN suppression on soils of similar properties in the UK. Furthermore, the positive correlation existing between Meldola's blue stain andTrehalose meth-

ods of PCN quantification shows that one could be a substitute for the other. It is therefore, recommended that Trehalose method be adopted as substitute for Meldola's blue stain method especially that it is easier to carry out and free from subjectivity. However, field experiment is needed to ascertain the recommendations.

Table 1: The results of ANOVA shows significant interactions between Nitrogen and Sulphur for both percentage viability and Viable Eggs Per Cyst, with 0.005 And 0.081 as *P* Values respectively.'R'Statistics also indicates slight correlation (0.3) between Meldola and Trehalose.

parameter	Meld vs Treh	V.E.C.	% Viability
P value N		0.374NS	0.0232*
P value S		0.0436*	0.397NS
P value NXS		0.081*	0.005**
HSD NXS		128.6	20.9
% CV		48.6	17.6
DF	78	76	76
R^2	0.09		
R	0.3		
Multiple R^2	0.1129		
RSE	9.927		
F-Statistic	9.922		
P-Value	0.002**		

KEY:
N = Nitrogen S = Sulphur NS = Not Significant DF = Degree Of Freedom
NXS = Nitrogen/Sulphur Interaction RSE = Residual Standard Error
V.E.C. = Viable Eggs Per Cyst Meld = Meldolatreh = Trehalose
HSD = Honestly Significant Difference CV = Coefficient Of Variation
** = Highly Significant * = Significant R = Correlation Coefficient

Figure 1: Percentage Viability of The PCN Eggs as Affected by the Treatments. Letters above the bars represent Tukey's Multiple Range Test. Treatments with the same alphabets are not significantly different among themselves. However, letters 'a', 'ab', and 'b' are significantly different among themselves.

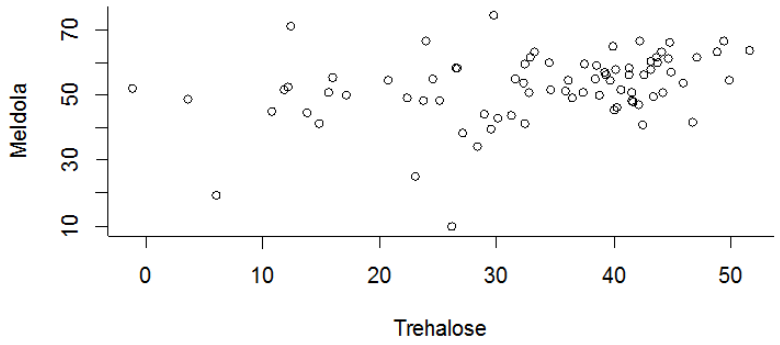

Figure 2: Relationship between the two methods of PCN Egg Viability Test with the dotted plot signifying slight (0.3) correlation betweenMeldola and Trehalose Methods.

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