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Determination of Nitrogen and Sulphur Ratio for Optimum Biomass and Glucosinolate Production in *Brassica juncea*(L.) for Biofumigationin the UK

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Abstract: *Glasshouse experiment was setup to find out the suitable ratio of sulphur and nitrogen for optimal biomass and glucosinolate production suitable for biofumigation using Brassica juncea (cv. Caliente 199).Sixteen treatment combinations were used and the parameters assessed include shoot and root biomass, leaf glucosinolate (GSL) level. Statistical analysis was conducted using GENSTAT software (17th edition). Results of two-way ANOVA showed significant interactions of the treatments on root biomass (p = 0.005). However, no interaction of the treatments was seen on the shoot biomass (p = 0.517). High Performance Liquid Chromatography (HPLC) was used to assess the amount of GSL in the composite leaf samples (to obtain its rough estimate, regardless of blocking), and the results showed variations between individual treatments. All the null hypotheses were rejected except for shoot biomass.Therefore, it is recommended that treatment ratio of 50kg/ha N and 60kg/ha S should be used for optimum biofumigationon soils of similar characteristics in the UK, due to its positive effects on brassica root biomass and GSL production.*

Keywords:*biomass, biofumigation, glucosinolate, Brasicajuncea, nitrogen and sulphur*

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I. Introduction

Biofumigation is the practice by which brassica cover crops such as oilseed radish (*Raphanussativus*) and Indian mustard (*Brassica juncea*) are grown to the flowering stage, chopped and incorporated into the soil for their abundant glucosinolates (GSLs). The macerated brassica tissues then release the GSLs which in the presence of an enzyme myrosinase and moisture further breakdown to form volatile compounds which suppress Potato Cyst Nematodes (PCN) in the soil (Back *et al*., 2015).Brassica crop is essentially a cool weather crop with variations in optimum temperature requirement below 21˚C and the family contains 350 genera with nearly 3,500 species, (Fourie *et al*., 2016). It releases GSLs which undergo hydrolysis in presence of the enzyme myrosinase and moisture to give rise to series of volatile compounds like isothiocyanates (ITCs), oxazolidine-2-thiones, epithionitriles, thiocyanates and nitriles. The product isothiocyanate is particularly identified as having a biocidal effect on PCN (Back *et al*., 2015). The mechanism could be achieved either through partial or full biofumigation. The former takes place in presence of growing brassica crop in conjunction with myrosinase-producing microbes, while the latter occurs after maceration and incorporation of the brassica plant (Ngala*et al*., 2015). The biocidal effects of ITCs are reported to interfere with vital physiological processes such as enzymatic, respiratory and nervous functions/physiology of the PCN (Ngala*et al*., 2015). The amount of GSLs and the consequent formation of ITCs depend on the biomass and species of brassica used, part of plant used, age of the plant, $_{P}$H and temperature of the medium and other microbial activities (Ngala*et al*., 2014).

The hydrolysis of the aliphatic glucosinolate in *B. juncea*gives rise to mostly ITCs at $_{P}$H 6-8 (Ngala*et al*., 2014). Moreover, brassicaceous crops are the most used in biofumigation process because of their relatively high content of GSLs, (Fourie *et al*., 2016). It is also reported that GSLs exuded from brassica roots can be hydrolysed into ITCs by soil fungi such as *Aspergillus spp* which can also produce the enzyme myrosinase (Ngala*et al*., 2014). This is termed as partial biofumigation.The need for biofumigation arises due to the ban imposed by UK government on red-band, class 1 plant protection products (PPP) and other nematicides such as aldicarb and methyl bromide. Besides, continuous use of such products leads to reduction in their efficacy due to increase in microbial populations which degrade the product active ingredients over time, (Fourie *et al*., 2016). Thus, identifying alternative means of combating the plant parasitic nematodes, other soil-borne pathogens, pests and weeds becomes crucial. The long practice of using members of the family Brassicaceae for controlling weeds, pests and pathogens remains potentially a vital alternative for farmers in the UK.

The efficacy of brassica in biofumigation is not restricted to PCN suppression but also shows suppression of lesion nematodes by 56 - 76% and that of other free-living nematodes by 99% (Fourie *et al*., 2016). Biofumigation can bring down the populations of soil pathogens, pests and weeds significantly, as put by Mattner (2008) who also observed that at neutral ₚH the volatile compounds released by brassicas are the like of active products resulting from degradation of synthetic fumigants such as dazomet, metam potassium and metam sodium, even though biofumigation is different from allelopathy. However, the allelopathic effect of brassica is also clearly demonstrated in the work of Aliki*et al*., (2014) where extracts from brassica roots, leaves, flowers and stems were found to significantly suppress root length, shoot length, seed germination and fresh weight of some weeds.

Different species of brassica produce different levels of GSLs due to their genetic differences. However, other factors such as diurnal cycle, sulphate and nitrogen exert significant influence on its concentration and toxicity, (Fourie *et al*., 2016). Mattner (2008) found that when root of *B. rapa* and *B. napus* were used they were six folds more effective than the shoots in suppressing *Rhizoctoniafragariae*. Martinez-Ballesta*et al*., (2013) also reported that higher concentration and diversity of GSLs in the roots than in the shoots of 29 brassica plants were documented. Furthermore, there are generally, a higher concentration of GSLs in younger leaves and reproductive tissues than there are in the mature leaves (Martinez-Ballesta*et al*.,2013). It is also reported that GSLs production reaches its maximum at mid flowering (Fourie *et al*., 2016).

Ngala*et al*. (2014) reported that the GSL concentration varied with season, with higher GSL concentrations recorded in summer-sown brassicas. A separate study conducted by Martinez-Ballesta*et al*., (2013) revealed that conditions such as low humidity, moderate temperatures, longer photoperiods and high light intensity (typical of spring/summer) triggered a higher accumulation of GSLs than autumn/winter condition. Ngala*et al*., (2014) also observed that the mortality rate of *Globodera pallida* was linearly related to the concentration of GSLs released in the medium. The amount of brassica biomass incorporated also contribute to the quantity of GSLs released (Fourie et al., 2016). The effectiveness of biofumigation also depends on whether the brassica produces aromatic or aliphatic GSLs.

Nikiforova, *et al*., (2003) reported that GSL was among the main sulphur containing compounds with 6% of the total sulphur in brassica contained in it. However, for optimum 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.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 production of optimum concentration of glucosinolate necessary for effective biofumigation. Therefore, the objectives of this research are:

To determine the correct proportion of sulphur and nitrogen to produce optimum biomass of Brassica juncea.

To determine the optimal proportion of sulphur and nitrogen for glucosinolate biosynthesis.

Null Hypotheses –

- Nitrogen and Sulphur proportion has no effect on the biomass of *Brassica juncea*
- Nitrogen and Sulphur proportion has no effect on glucosinolate biosynthesis

II. Methodology

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 ₚH was 6.3 and the nitrogen/sulphur status of the soil was determined prior to mixing and pot-stocking of the soilusing macro-kjeldahl/turbidometric methods and found to be 0.35/0.025% respectively. Automated irrigation facility was used.Ammonium nitrate (Nitram®) (NH_4NO_3) 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 (Table 2) 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 on 23rd May 2017, 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 4th week after sowing (WAS). At 5 WAS, most of the plants attained 35% florets. At the end of 6th week the plants were carefully uprooted from the pots. The shoots and the roots were separated using secateurs and the soil washed off the root, blotted with tissue paper before their separate biomass was recorded.

Representative leaf samples from each pot were sealed in polythene bags, labelled and taken to laboratory immediately, and stored for two weeks at -80ºC before freeze-drying. The samples were latter freeze-dried for five days, weighed, and milled as composite samples, based on experimental treatments. Part of the milled and freeze-dried leaf samples were extracted and analysed usingHigh Power Liquid Chromatography (HPLC). The procedures adopted were a slight modification of that used in Ngala*et al*., (2014), i.e. extraction in cold methanol as adapted in Doheny-Adams *et al*., (2017).Sixteen small glass pipettes were vertically arranged in a stand as ion-exchange column, with glass wool plugs in their neck. 0.5ml of a well-mixed suspension of ion-exchange resin (Acetic Acid) was put into each pipette and allowed to drain into waste collection tray. 2ml of imidazole formate was used to rinse the pipettes followed with 1ml portions of distilled water (twice), while allowing complete drainage into the waste tray at each rinsing.Sixteen polypropylene tubes were labelled T1, T2, ---T16, and set on the rack. 0.2g of composite samples (weighed to the nearest 0.01g) were placed in their respective tube. 4.0 ml of cold methanol was added to each tube and the tube was pressed on to a spin for thorough mixing. The tubes were placed on a shaker to swirl for 3 min and put to centrifuge at 5,000 rpm for 3 min. Then, supernatant liquid from tubes T1, T2, ---T16, were transferred into another set of tubes labelled T1*, T2*, ---T16*. The process was repeated for tubes T1, T2, ---T16, containing the solid residues and the second supernatants were combined with the supernatant liquids in T1*, T2*, ---T16*. 1.0ml of each combined extract was transferred into a corresponding ion-exchange column (without disturbing the resin) and allowed to drain into the waste tray. 1.0ml portions of sodium acetate buffer $_{p}$H 4.0 were gently added and allowed to drain into the waste collection tray after each addition. 0.75ml (75µl) of diluted purified sulfatase solution was added to each column, followed by 2ml (200µl) of internal standard (IS). The top of the pipettes (columns) were sealed with parafilm and allowed to react overnight at ambient temperature. The next day, glass vials were labelled appropriately and placed under each ion-exchange resin column to collect the eluate. The desulfo-glucosinolate obtained were eluted with three 0.33ml portions of deionised water by allowing the water to completely drain into the vial after each addition. The eluate was gently homogenised and taken for HPLC analysis.

Loading of the vial tubes containing the samples was done in such a way that the first three tubes contain blank sample, sinigrin and glucotropaeolin respectively, then followed by those containing the eluate arranged chronologically on the autosampler. The HPLC was set in operation and took about 30min to read and produce the peak areas of each sample. The amount of GSL (µmol g^{-1}) was calculated from equation 1:

$$\mathrm{GSL} = \left(\frac{\mathrm{Ag}}{\mathrm{As}}\right) \times \left(\frac{\mathrm{n}}{\mathrm{m}}\right) \times \ \mathrm{RRF} \qquad (1)$$

Where A_g = Peak Area of the Relative Glucosinolate; A_s = Peak Area of the IS, n = Amount of IS (µmol), m = Mass of Test Sample (g) and RRF = Relative Response Factor of GSL

The parameters assessed include shoot and root biomass and GSLs content using HPLC. Two-way ANOVA was used to analyse the effect of treatments on the shoot and root biomass using GENSTAT package. Tukey's multiple range test was also conducted to determine the variations between individual treatments. However, simple bar plot was used to show the rough estimates of the GSL in the leaf samples because the milling was carried out as composite, thus, there was no blocking.

III. Results

The two-way ANOVA shows there were no significant differences in the roots (p = 0.299 and 0.187) or shoot (p = 0.186 and 0.230) biomass for nitrogen and sulphur applied as individual treatment. While on one hand no significant interaction existed between the treatments on the shoot biomass (p = 0.517), on the other hand, a significant interaction was observed between nitrogen and sulphur on the root biomass, p = 0.005,(Table 3). Tukey's multiple range test carried out on the root biomass showed no differences among values with the same letters, but significant differences exist between value with letter 'b' and others with letters 'a' or 'ab' (Table 3). However, Tukey's test was not possible on shoot biomass where there was no significant interaction. The percentage coefficient of variation (units) for both shoots and roots are 14.1 and 22.7 respectively.The level of glucosinolates contained in each of the composite leaf samples (ran through the HPLC without blocking) is pre-

sented in Figure 1. Simple descriptive statistics (bar graph) was used to get the rough idea of how each treatment label contributes to the production of GSL. The graph shows that treatment fourteen (50 N / 60 S) produced the highest level of GSL (17.05 µmolg^{-1}), while treatment five (0 N / 20 S) gives the lowest amount of GSL (2.28 µmolg^{-1}). However, the later value is even lower than the glucosinolate produced in the control (6.98 µmolg^{-1}).

IV. Discussions

It is clear from Table 3 that nitrogen or sulphur as individual treatment has no significant effect on root or shoot biomass, but when the two are combined (in the right proportions) they produced significant interactive effect ($p = 0.005$) on the root biomass. This concurred with the work of McGrath *et al*., (2009) who found that the significant interaction ($p< 0.05$) between N and S resulted in seed yield and seed oil content of oilseed rape by 58%. The non-significant interaction effect observed on the shoot biomass could be due to improper translocation of sulphur (applied as raw pellets in elemental form) within the short duration of the experiment or due to allocation of S towards secondary metabolites rather than shoot growth physiology because, the result disputes the finding of Van der Kooij*et al*., (2008) who observed a significant difference ($p<0.05$) in the interactions between N and S in the dry matter of Arabidopsis thaliana shoots (biofumigant different from *B. juncea*). However, Mattner (2008) and Martinez-Ballesta*et al*., (2013) found that root biomass exerts higher PCN suppressive effects (by six folds) than shoot biomass in biofumigation processes.

The highest amount of GSL produced by treatment fourteen (17.05 µmolg^{-1}) may be due to the high quantity of S contained in the treatment (50 N / 60 S); and Nikiforova*et al*., (2003) reported that GSL is a S containing compound with 6% of the total sulphur in brassica contained in the GSLs. Martinez-Ballesta*et al*., (2013) also reported that low N supply together with high S fertilization produced an increased concentration of GSLs in broccoli; and this agrees with the result in treatment fourteen (50 N / 60 S). In the same vein, the result agrees with the work of Zhao *et al*., (1993) who observed an increase in GSL content of the seed of oilseed rape when N was applied in combination with S than without S. Interestingly, the same treatment that gave the highest root biomass (11.14g) also showed a corresponding highest production of GSL (17.05 µmolg^{-1}). This agrees with the work of Fourie *et al*., (2016) who reported that he amount of brassica biomass incorporated also contribute to the quantity of GSLs released. However, the GSL produced in treatment five (2.28 µmolg^{-1}) is even lower than that produced in the control (6.98 µmolg^{-1}). This could be due to microbial activities in the medium which could lead to immobilisation of the little sulphur applied (in treatment five) as explained by Brady *et al*., (1999).

V. Conclusion

The significant interactions recorded on root biomass ($p =0.005$) and the variation in the GSL contents of the leavesshowed that the null hypotheses formulated (except for shoot biomass) are rejected.It is clear then that proportions of N and S, have effect on the parameters assessed. However, the N and S effects on shoot biomass need to be revisited. Therefore, based on this research the N and S proportion recommended for optimum biofumigation practice on soils of similar characteristics in the UK is 50 kg/ha N and 60 kg/ha S because of their positive effect on root biomass and GSL production. However, repeated field experiment is also required to ascertain the applicability of the results.

Table 1: Fertilizer recommendations (kg/ha) used in calculating the treatment per pot

Nitram (kg/ha)	0	50	100	150
Sulphur (kg/ha)	0	20	40	60

Table 2: Nitrogen/Sulphur Treatment Combinations per pot as Calculated from the Nutrient Recommendations.

Treatment	**Recommendation (kg/ha)**	**Nitrogen/Pot (g)**	**Sulphur/Pot (g)**
1	0.00 N + 0.00 S	0.00	0.00
2	50.0 N + 0.00 S	0.66	0.00
3	100 N + 0.00 S	1.31	0.00
4	150 N + 0.00 S	1.97	0.00
5	0.00 N + 20.0 S	0.00	0.09
6	50.0 N + 20.0 S	0.66	0.09
7	100 N + 20.0 S	1.31	0.09
8	150 N + 20.0 S	1.97	0.09
9	0.00 N + 40.0 S	0.00	0.18
10	50.0 N + 40.0 S	0.66	0.18
11	100 N + 40.0 S	1.31	0.18
12	150 N + 40.0 S	1.97	0.18
13	0.00 N + 60.0 S	0.00	0.27
14	50.0 N + 60.0 S	0.66	0.27
15	100 N + 60.0 S	1.31	0.27
16	150 N + 60.0 S	1.97	0.27

Table 3: Means for the effects of the sixteen treatment combinations on the fresh brassica root and shoot biomass. The letters in front of the values indicate Tukey's multiple range test. Treatments with the same letter or letters combination show no significant difference among themselves.

Treatment (kg/ha)	Foliage fresh weight (g)	Root fresh weight (g)
0 N / 0 S	126.4	8.42ab
50 N / 0 S	113.4	8.14ab
100 N / 0 S	132.4	8.16ab
150 N / 0 S	130.7	7.56ab
0 N / 20 S	126.7	7.24ab
50 N / 20 S	113.7	6.68a
100 N / 20 S	127.2	8.06ab
150 N /20 S	117.7	6.76a
0 N / 40 S	119.8	9.30ab
50 N / 40 S	100.6	5.94a
100 N / 40 S	123.5	6.92a
150 N / 40 S	114.2	7.70ab
0 N / 60 S	113.9	7.94ab
50 N / 60 S	128.1	11.14b
100 N / 60 S	119.7	7.06ab
150 N / 60 S	122.5	6.92a
P value N	0.186	0.299
P value S	0.230	0.187
P value N X S	0.517	0.005*
SED N	5.37	0.557
SED S	5.37	0.557
SED N X S	10.74	1.113
% CV	14.1	22.7

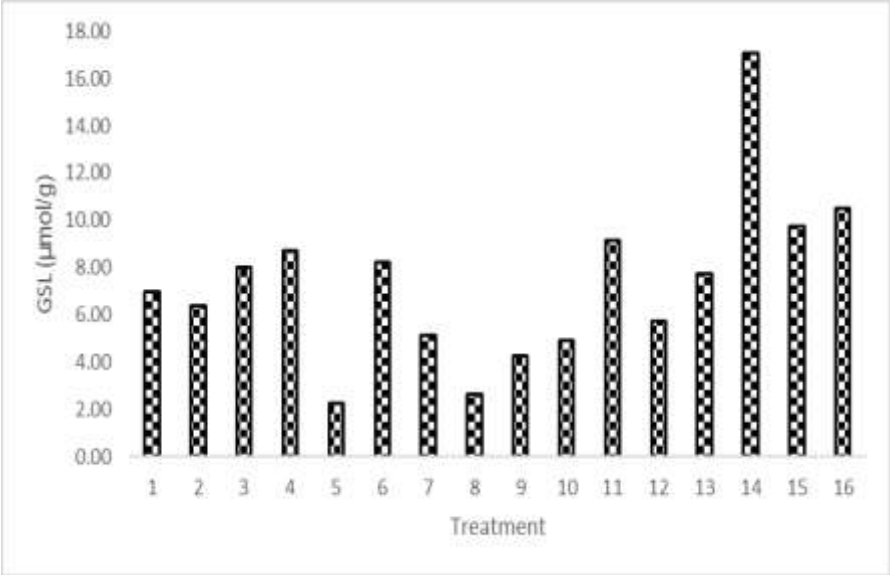

Figure 1: Effects of Treatments on glucosinolate content (µmolg^{-1}) of the composite leaf samples as detected by HPLC

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