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Potential isothiocyanate release remains constant across biofumigant seeding rates

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Abstract

Biofumigation is an integrated pest management method involving the mulching of a glucosinolate containing cover crop into a field in order to generate toxic isothiocyanates, effective soil borne pest control compounds. Variation in biofumigation efficacy demonstrates a need to better understand the factors affecting pest control outcomes and develop best practice for biofumigant choice, growth conditions and mulching methods which allow the greatest potential isothiocyanate release. We measured the glucosinolate concentration of 6 different commercial varieties of three different biofumigant plant species: *Brassica juncea* (ISCI99, Vitasso, Scala) *Raphanus sativus* (Diablo, Bento) and *Sinapis alba* (Ida Gold). Plants were grown at a range of commercially appropriate seeding rates and sampled at three growth stages (early development, mature, and 50% flowering). Within biofumigant species, highest ITC release potential was achieved with *B. juncea* cv. ISCI99 and *R. sativus* cv. Bento. Highest ITC release potential occurred at 50% flowering growth stage across species. Seeding rate had minor impact on ITC release potential from *R. sativus* but had no significant effect on the ITC release potential of *B. juncea* or *S. alba* cultivars.

Introduction

Biofumigation is an integrated pest management method involving the mulching of a brassicaceae cover crop into agricultural fields causing a release of toxic secondary metabolites and reduction in soil borne plant pests *(1)*. Aliphatic and aromatic glucosinolates (GSLs), sulphur rich compounds found almost exclusively in brassicaceae, are hydrolysed and transformed to short-lived, highly reactive isothiocyanates upon plant disruption *(2, 3)*. Isothiocyanates (ITCs) are thought to be the primary active ingredient in biofumigation and their toxicity has been demonstrated for a broad range of soil borne pathogens *(1)*. However, it is important to note that complete conversion of GSLs to ITCs by mulching is unlikely, with some researchers questioning whether the final ITC dose is sufficient for pest suppression on its own *(4)*. In addition to isothiocyanate release, other changes resulting from biofumigation, including soil microbial community shifts *(5)*, enhanced nutrient cycling, and production of other compounds such as dimethyl disulphide and dimethyl sulphide *(6)* may also play a role in pest suppression.

Quantification of isothiocyanates is analytically challenging and therefore glucosinolate concentrations in plant tissues have been used as a proxy to estimate potential isothiocyanate release in the field. Kirkegaard and Sarwar (1998) examined variation in biomass and glucosinolate profiles of 80 different brassicas to explore their possible use as biofumigants and found significant variation in GSL field potential (i.e. the concentration of glucosinolates per field area) ranging from 0.8 to 45.3 mmol m^{-2} *(7)*. GSL field potentials alone may be misleading however, since glucosinolate profiles (the types and relative amount of glucosinolates produced) vary between species and determine the type and quantity of ITC release which determines the overall biofumigation effect *(1)*. For instance, *Brassica napus* mainly produces indole glucosinolates which do not form ITCs, while other species, such as *Brassica juncea* and *Sinapis alba,* predominantly produce aliphatic and aromatic glucosinolates respectively *(7)*. Additionally, biofumigant selection must also take into

account varying GSL content between tissue types. Roots, which contribute on average 23.6% of all plant glucosinolates, often contain the majority of indole glucosinolates and are generally harder to macerate likely contributing to a slower release of ITC *(7)*.

Biofumigation methods often macerate plant tissues at the 50% flowering stage. While GSL profiles remain relatively stable within plant species *(7)* they can vary throughout the plants lifecycle *(8)*. For instance, glucosinolate concentrations in seed are not correlated with glucosinolate concentrations in root and shoot tissue *(7)* and GSL concentrations and content are generally lower in younger plants *(8)*. Seasonal and diurnal cues also affect glucosinolate content in plant tissues. Biosynthesis of glucosinolates in *Arabidopsis* was shown to increase rapidly in response to light *(9)*, suggesting that highest levels of GSLs occur during mid-day and that best practice for biofumigation would avoid incorporation in the early morning. In addition, higher glucosinolate concentrations have been reported for biofumigants grown in spring rather than autumn *(1)*.

Glucosinolate variations due to species, tissue type, plant age, season and time of day complicate predictions on the effectiveness of biofumigants, which is further compounded by differences in resulting ITC efficacy. A further factor, biofumigant plant seeding rate, or plant density, has not yet been studied in the context of GSL content. Plant density is known to affect yield *(10)*, photosynthesis *(11)*, and phytochemical production *(12)* which are all likely to play a role in the biofumigation effect. Seeding rates are also established as having an impact on plant physiology. For example, in *B. napus*, planting density has recently been shown to affect lignin production *(13)*. Despite the effects plant density can have on plant development and physiology, no studies have yet examined the effect of plant density on glucosinolate production. In addition, the combined effect of ontology, plant density and plant tissue on the overall GSL concentration is unknown as these processes have not been studied together. Not only do biofumigant species' biological parameters determine ITC production, but measurements rarely take into account environmental drivers such as soil pH, nutrient loading, soil type and climatic conditions, all of which may contribute to variable

results in field trials. We can control to some extent the glucosinolates produced but this may not translate to predictable performance in the field: variability in biofumigant efficacy has been noted between field trials examining the same pathogen and biofumigant *(1)*. ITCs in laboratory toxicity experiments do not necessarily translate to field outcomes. ITCs vary in half life, reactivity, volatility, and percent sorption to organic matter, causing complex behaviours in soil and variable effective toxic doses dependent upon both environmental variables (soil type and density, water content, temperature) and biological variables (plant species, season, growth stage, and tissue type). For example, while aromatic ITCs are more toxic in agar plate experiments *(14)*, their activity in soils is suppressed to a much greater degree than aliphatic ITCs such as allyl-ITC due primarily to sorption to organic matter *(15)*.

Variation in the biofumigation efficacy results of field studies using the same or similar biofumigant demonstrate a need to develop consistent practice for biofumigant choice (for GSL type), growth conditions and mulching methods which will allow the largest potential ITC release. In order to work towards developing such a consistent practice, we measured the glucosinolate concentration of six different commercial biofumigants within 3 different plant species: *Brassica juncea* (ISCI99, Vitasso, Scala) *Raphanus sativus* (Diablo, Bento) and *Sinapis alba* (Ida Gold), sown at a variety of seeding rates and sampled at 3 growth stages (early development, 50% maturity, and 50% flowering). This study aims to clarify the following points:

(1) Which cultivar has maximum GSL release potential among a sub-set of commercial cultivars?

(2) Can we confirm that maximum field GSL content is reached at the 50% flowering stage.

(3) What is the optimal seeding rate for maximum ITC release potential?

Results

Effect of seeding rate on biofumigant biomass

Commercial mustard cultivars: *S. alba* (cv. Ida Gold) and *B. juncea* (cv. Scala, cv. ISCI99, cv. Vitasso) were planted at 4 seeding rates spanning the range recommended by seed suppliers: 6, 8, 10, and 12 kg/ha. They were harvested once 50% of the plants had flowered. Total above-ground biomass for the mustards ranged from 24 tonnes/ha (for Ida gold) to 50 tonnes/ha (for Vitasso) (table 1). There was a significant effect of mustard cultivar on total biomass, seeding rate on total biomass and a combined significant effect of cultivar and seeding rate on total biomass (ANOVA, $p<0.001$ supplementary table 1.1). For a seeding rate range of 8-12 kg/ha there was no significant effect of seeding rate on the total above-ground biomass of the mustard cultivars, however biomass was significantly lower in mustard cultivars grown at a seeding rate of 6 kg/ha (table 1, supplementary table 1.1.1). Mustard leaf biomass accounted for 40% - 50% of total aboveground shoot biomass, which ranged from an average of 27 (Ida gold) to 43 (Vitasso) tonnes/ha across seeding rates. Ida Gold had a significantly (~27%) lower total biomass and ~32% lower stem biomass than ISCI99, Vitasso and Scala. Total biomass of between *B. juncea* cultivars did not differ significantly except for a slight but significantly higher biomass for Vitasso compared with ISCI99 (table 1, supplementary table 1.1.2).

R. sativus cultivars (cv. Diablo and cv. Bento) were planted at 3 commercially suggested seeding rates: 10, 15 and 20 kg/ha, and harvested once 50% of the plants had flowered. Biomass ranged from 62 to 74 tonnes/ha for Diablo and 52 to 71 tonnes/ha for Bento and was positively correlated with seeding rate (table 1). There were significant effects of tissue type and seeding rate on biomass as well as a significant interaction effect between tissue type and seeding rates on biomass (supplementary table 1.2). Stem biomass was generally lower than leaf biomass and the increase in total biomass at higher seeding rates was due primarily to an increase in stem biomass which grew from 15 tonnes/ha (10 kg/ha seeding rate) to 35 tonnes/ha (20 kg/ha seeding rate) (table 1). At the highest seeding density leaf biomass accounted for ~50% of total biomass (table 1). There was no significant effect of radish cultivar on biomass. (table 1, supplementary tables 1.2 to 1.2.2).

Species	Cultivar	Seeding rate	Stem biomass (tonnes/ha ± st.dev)	Leaf biomass (tonnes/ha ± st.dev)	Total above-ground biomass (tonnes/ha ± st.dev)
B.juncea	ISCI99	6 kg/ha	15.1 ± 3.7	14.2 ± 3.8	29.3 ± 5.6
		8 kg/ha	22.6 ± 5.3	17.1 ± 4.5	39.7 ± 7.7
		10 kg/ha	24.6 ± 3.5	18.5 ± 3.9	43.1 ± 2.6
		12 kg/ha	18.8 ± 1.5	19.5 ± 1.4	38.3 ± 2.4
	Scala	6 kg/ha	23.5 ± 5	13.8 ± 2.4	37.3 ± 7.4
		8 kg/ha	19.2 ± 1.6	13.5 ± 2.2	32.7 ± 3.0
		10 kg/ha	23.5 ± 1.6	13.3 ± 2.5	36.8 ± 3.5
		12 kg/ha	28.3 ± 3.4	16.3 ± 5.0	44.7 ± 6.2
	Vitasso	6 kg/ha	19.3 ± 3.7	14.1 ± 4.1	33.4 ± 7.5
		8 kg/ha	30.2 ± 6.1	20.1 ± 4.7	50.3 ± 10
		10 kg/ha	29.0 ± 3.4	20.7 ± 2.7	49.7 ± 5.2
		12 kg/ha	23.5 ± 2.7	16.7 ± 2.8	40.2 ± 5.3
S. abla	Ida Gold	6 kg/ha	12.7 ± 1.8	11.3 ± 1.3	23.9 ± 2.7
		8 kg/ha	15.4 ± 1.7	16.2 ± 5.1	30.4 ± 8.1
		10 kg/ha	15.4 ± 1.7	10.7 ± 3.5	26.1 ± 3.2
		12 kg/ha	13.8 ± 2.2	12.6 ± 2.1	26.4 ± 4.0
R. sativus	Bento	10 kg/ha	18.1 ± 0.17	26.3 ± 1.9	51.7 ± 2.3
		15 kg/ha	28.5 ± 8.0	27.6 ± 3.6	62.6 ± 11
		20 kg/ha	32.3 ± 2.2	32.9 ± 6.0	71.1 ± 5.9
	Diablo	10 kg/ha	14.7 ± 1.4	36.9 ± 7.1	62.2 ± 12
		15 kg/ha	24.8 ± 3.0	38.0 ± 3.4	70.9 ± 7.2
		20 kg/ha	35.0 ± 1.5	32.6 ± 2.9	74.4 ± 3.9

Table 1: Mean leaf, stem and total above-ground biomass for various commercial biofumigants grown at different seeding densities (n=6, biological repeats, except R. sativus where n=3). Results from statistical analyses can be found in supplementary tables 1.1 to 1.2.2.

Glucosinolate concentration

Glucosinolate concentration of leaf and stem tissue was assessed for each cultivar at the maximum and minimum seeding rates (6 and 12 kg/ha for *S. alba* and *B. juncea*, and 10 and 20 kg/ha for *R.*

sativus) at three growth stages: rapid growth, 50% maturity, and 50% flowering. Sinigrin made up more than 90% of total glucosinolate content in green tissues of *B. juncea* cultivars with higher concentrations found in the leaves (fig 1). There was a significant effect of cultivar and a significant combined effect of cultivar and tissue type on sinigrin concentration: cultivars differed significantly in leaf sinigrin concentrations but not in stem sinigrin concentrations (supplementary tables 2.1 to 2.2). Sinigrin concentrations in ISCI99 leaves were on average ~12% and ~29% higher than in Scala and Vitasso leaves respectively. There was a significant effect of seeding rate on sinigrin concentrations which were on average 20% higher at the lower rate of 6kg/ha (fig 1, supplementary table 2.1). Sinigrin concentrations were modulated by growth stage with mean concentration in leaves significantly higher at 50% flowering relative to both other growth stages and mean concentration in the stems significantly lower at 50% flowering relative to the rapid growth stage (fig 1, supplementary table 2.3).

FIGURE 1

Figure 1: Sinigrin concentrations in field grown leaves and stems of *B. juncea* cultivars (ISCI99, Scala and Vitasso) sampled during rapid growth, at 50% maturity and 50% flowering. Error bars represent standard error (n=3-4, biological repeats). Results from statistical analyses can be found in supplementary tables 2.1 to 2.1.3.

S. alba (Ida Gold) does not produce the aliphatic glucosinolate sinigrin in appreciable amounts. Glucotropaeolin and sinalbin are both aromatic glucosinolates and accounted for over 90% of the total glucosinolate content in the green biomass of *S. alba* (cv. Ida gold) (fig 2). A significant statistical three way interaction was observed between the effects of tissue type, seeding rate and growth stage on total glucosinolate concentration (ANOVA: $F_{(2,\ 32)}=5.22$; $p=0.011$). Total glucosinolate concentration was significantly higher in leaves in all conditions (fig 2, supplementary tables 2.2 to 2.2.4). Leaf glucosinolate concentration was higher at the 50% flowering stage (~45 $\mu mol.g^{-1}$) than the rapid growth stage (~30 $\mu mol.g^{-1}$) and stem glucosinolate concentration decreased with plant growth stage (from approximately 12 $\mu mol.g^{-1}$ at rapid growth to 5 $\mu mol.g^{-1}$ at

50% flowering) (fig 2, supplementary table 2.2.1). On average, the differences and relative contributions of glucosinolate concentrations in the leaf and stem increased over time. Glucosinolate concentrations were significantly higher at higher seeding rates only for leaves sampled from plants at 50% maturity (supplementary table 2.2.3).

FIGURE 2

Figure 2: Glucotropaeolin and sinalbin concentrations in field-grown leaves and stems of *S. alba* (cv. Ida Gold) sampled during rapid growth, at 50% maturity and at 50% flowering. Error bars represent standard error (n=3-4). Results from statistical analyses can be found in suplimentary tables 2.2 to 2.2.4.

Glucoraphenin and glucoraphasatin are both aliphatic glucosinolates and account for over 90% of ITC releasing glucosinolates in *R. sativus* (cv. Diablo and cv. Bento) shoots. Variability of glucosinolates within sample sets was much higher than with the mustards (fig 3). Concentrations of glucoraphenin were significantly higher in Bento than in Diablo, and in leaves than in stems (ANOVA: $F_{(1, 64)}$ = 9.143; p<0.01; and ANOVA:$F_{(1,64)}$ =54.164; p<0.001 respectively)(supplementary table 2.3.1). A significant effect of growth stage on glucoraphenin was also identified (ANOVA:$F_{(2,64)}$=3.521; p=0.035). There was a three way interactive effect of growth stage, tissue type and seeding rate on glucoraphasatin concentrations (ANOVA:$F_{(2,64)}$=3.823; p=0.027) that were significantly lower in leaves sampled at 50% maturity from plants at 20kg/ha relative to stems sampled at rapid growth at 10kg/Ha (supplementary tables 2.3.2 and 2.3.2.1). No interactive effect of any combination of seeding rate, growth stage, cultivar, and tissue type on glucoraphenin concentration was detected. Total glucosinolate concentrations were significantly higher in Bento than in Diablo and in leaves than in stems (ANOVA:$F_{(1, 64)}$=5.453; p=0.023; and ANOVA:$F_{(1, 64)}$=15.05; p<0.001 respectively) and a significant effect of growth stage on total glucosinolate concentrations was also identified (ANOVA:$F_{(2,62)}$=4.143, p=0.020)(Supplementary table 2.3.3). The glucosinolate concentrations from radish plants sampled at the rapid growth stage were significantly higher than total glucosinolate concentrations from radish plants sampled at the 50% maturity stage (TukeyHSD, p adj=0.016). No

interactive effect of any combination of seeding rate, growth stage, cultivar and tissue type on total glucosinolate concentration was found (Supplementary table 2.3.3)

FIGURE 3

Figure 3: Glucoraphasatin and glucoraphanin concentrations in field grown leaves and stems of *R. sativus* (cv. Bento and cv. Diablo) sampled during rapid growth, at 50% maturity and at 50% flowering. Error bars represent standard error (n=3-4). Results from statistical analyses can be found in supplementary tables 2.3 to 2.3.3.3.

Glucosinolate concentration in the field.

The total glucosinolate concentration expected per area of field at 50% flowering (i.e. the typical stage at which the biofumigants are incorporated) was assessed across the biofumigant cultivars and least/greatest experimental seeding rates. For *B. juncea* cultivars, total sinigrin concentration in the field ranged from 16 to 24 mmol/m^2. Both cultivar and seeding rate individually had significant effects on the concentration of sinigrin in the field (ANOVA:$F_{(2, 18)}$= 6.36; $p<0.01$ and ANOVA:$F_{(1, 18)}$=4.55; $p<0.047$ respectively)(supplementary table 3.1). Fields in which ISCI99 was sown at a seeding rate of 12kg/ha contained a significantly higher glucosinolate concentration than fields in which Scala and Vitasso were sown at the same rate (fig 4)(supplementary table 3.1.1). No interactions were found between cultivar and seeding rates on glucosinolate field concentration in *B. juncea* cultivars (supplementary table 3.1). For the *S. alba* cultivar (Ida Gold), mean glucosinolate concentrations ranged from 1.4 mmol/m^2 to 1.6 mmol/m2 and 9.2 mmol/m^2 to 11 mmol/m^2 for glucotropaeolin and sinalbin respectively. Seeding rate had no effect on glucosinolate field concentration, but total glucosinolate concentration per area of field was significantly lower in fields

growing Ida Gold at a seeding rate of 6kg/ha than in fields growing the *B. juncea* cultivars (supplementary table 3.2.2). Diablo (*R. sativus*) mean glucosinolate concentrations ranged from 13 to 17 mmol/m^2 and 16 to 18 mmol/m^2 for glucoraphasatin and glucoraphenin respectively (fig 5). Bento (*R. sativus*) mean glucosinolate concentrations ranged from 5.4 to 14 mmol/m^2 and 28 to 31 mmol/m^2 for glucoraphasatin and glucoraphenin respectively. No significant difference in total glucosinolate concentrations was identified between the cultivars or seeding rates for *R. sativus*, but concentrations of glucoraphenin were signficantly higher in Bento than in Diablo (ANOVA:$F_{(1,\ 11)}$= 5.316; p=0.042)(supplementary table 3.4).

FIGURE 4

Figure 4: (A) Mean concentrations of sinigrin per area of field growing *B. juncea* (ISCI99, Scala, and Vitasso) seeded at rates of 6 kg/ha or 12 kg/ha and (B) mean concentrations of glucotropaeolin and sinalbin per area of field growing *S. alba* (Ida Gold) seeded at rates of 6 kg/ha or 12 kg/ha. Error bars represent standard error (n=3-4) Results from statistical analyses can be found in supplementary tables 3.1 to 3.2.2.

FIGURE 5

Figure 5: Mean glucosinolate concentrations per area of field growing *R. sativus* (Bento and Diablo) drilled at rates of 10 kg/ha or 20 kg/ha. Error bars represent standard error (n=3-4). Results from statistical analyses can be found in supplementary tables 3.3 to 3.4.1.

Discussion

Which commercial biofumigant cultivar has the highest ITC release potential?

The biofumigation effect of cultivars examined in this study depends on both the type and amount of ITC released at incorporation. The three species examined have entirely different glucosinolate

profiles, but the profiles of cultivars within those species did not differ. In the following discussion it is important to note that direct comparisons between total glucosinolate concentrations to assess biofumigation potential are informative within species, but given that ITCs differ in their toxicity and volatility, it is difficult to directly compare biofumigation potential between species. In addition, it should be noted that typically dryer summer soils are likely to have an effect on both the GSL to ITC conversion, microbial degradation as well as ITC volatility *(1, 5).*

The cultivar with the highest ITC release potential was *R. sativus* Bento which produced ~45 mmol.m^{-2} glucosinolate at a drilling rate of 20 kg/ha and at 50% flowering, compared to ~31 mmol. m^{-2} for *R. sativus* Diablo. *R. sativus* has been reported to control populations of the potato cyst nematode *Globodera pallida (16)*. Hansen and Keinath (2013) compared ITC release from incorporation of *R. sativus* and *B. juncea L.* in two field trials and detected relatively low ITC release for *R. sativus* in the first trial and no ITC release in the second *(17)*. In this study, glucosinolate concentrations in *R. sativus* were more variable within sample sets than glucosinolate concentrations in *S. alba* and *B. juncea*. Variability in GSL production, hence the biofumigation potential of *R. sativus* limits its appropriateness as a biofumigant candidate because uniform and replicable outcomes are desirable. In addition, the two major glucosinolates identified in *R. sativus,* glucoraphenin and glucoraphasatin, are hydrolysed to isothiocyanates which are reportedly less volatile and toxic (with an 2-fold increase in LD90 for the soil-borne fungal pathogen *Verticillium dahliae*) than the smaller chain allylisothiocyanate formed from hydrolysis of sinigrin, the primary glucosinolate in *B. juncea (18)*.

Only one cultivar of *S. alba* was examined in this study: Ida Gold. Incorporation of *S. alba* in field trials is reported to reduce at least one fungal species: *Aphanomyces euteiches (19)*. The majority glucosinolates in Ida Gold *S. alba* green tissue at all growth stages were aromatic glucosinolates: sinalbin and glucotropaeolin. Aromatic ITCs are reported to have higher contact toxicity but lower volatility than aliphatic ITCs *(20)*. Studies comparing relative toxicity of aromatic to aliphatic ITCs in both laboratory and field tests report that despite higher toxicity of aromatic ITCs in contact *in vitro*

experiments, short chain aliphatic ITCs are more effective in field conditions *(15)*. In this study, total mean glucosinolate concentration per area of field is lower in *S. alba* Ida Gold than *B. juncea* ISCI99 and field toxicity of the corresponding ITCs post mulching is also likely to be lower for Ida Gold.

B. juncea ISCI99 fields produced higher glucosinolate concentrations (24 $mmol.m^{-2}$ glucosinolate at a drilling rate of 20 kg/ha and at 50% flowering) than *B. juncea* Scala and *B. juncea* Vitasso (~17 $mmol.m^{-2}$ and ~16 $mmol.m^{-2}$ respectively). Incorporation of *B. juncea* in field trials is reported to control plant parasitic nematode species including: *Tylenchus semipenetrans (21)*, *Meloidogyne chitwoodi (22)*, *Meloidogyne javanica (23)*, *Globodera pallida (16)*, and fungal species: *Sclerotinia minor* (21), *Rhizoctonia solani (24)*. However, other studies have reported no effect of *B. juncea* incorporation on some of these same species *(25, 4, and 1)*. As a result of this variability in efficacy, the major glucosinolate found in *B. juncea* cultivars, namely sinigrin, has been the subject of many studies relating to biofumigation. Allylisothiocyanate (AITC), a product of sinigrin hydrolysis, is often reported to be one of the most toxic naturally occurring isothiocyanates by virtue of its short side chain and high volatility *(1)*. Sarwar et al. (1998) reported that AITC and methyl isothiocyanate were similarly effective in their ability to suppress mycelial growth of five root pathogens in vapour exposure headspace experiments *(20)*. Depending on the plant and type of control required, an estimated 517 to 1294 nmol/g soil of methyl ITC is required for soil sterilisation *(26)*. Our results indicate a maximum AITC potential of 16-24 $mmol/m^2$ which, assuming a soil bulk density of 1.4 g cm^{-3} and incorporation to 20 cm, would give a considerably lower maximum of 85 nmol/g. In addition, given that ITC production is dependent on soil conditions, incorporation depths can be substantially deeper, and glucosinolates are unlikely to be fully converted to ITC, true ITC concentrations following incorporation are likely to be even lower *(27)*. In addition, methyl ITC is reported to have higher activity than AITC in vapour exposure experiments and in soil experiments *(15)*. While initial AITC release at these levels is not enough to completely sterilise the soil, soil pathogen control observed in numerous biofumigation studies may result from a cocktail of toxic chemicals (including DMS) to which AITC concentrations contribute (6).

When should biofumigants be incorporated for maximum ITC release potential?

It has been reported that *B. napus* rapidly degrades glucosinolates during flowering *(28)*, causing concern that the typical biofumigant incorporation time, i.e. once half the crop has begun to flower, may not be optimal for maximum ITC release. For the mustards *B. juncea* and *S. alba*, glucosinolate concentrations in the leaves as well as plant biomass were highest at the 50% flowering stage indicating that the maximum ITC release potential across the three growth stages studied, and advised time of incorporation, remains when half the crop has flowered. These results are in agreement with other published data for mustards which indicate that the maximum glucosinolate concentration occurs at the later growth stages *(7)*. However, it should be noted that according to another study, GSL concentrations were highest prior to flowering *(29)*. A further study reported highest glucosinolate concentrations at very early growth stages of *S. alba*, but this study examined cotyledons at a growth stage that would be unrealistic to consider for biofumigation incorporation *(30)*. For *R. sativus* cultivars, mass-dependent concentration of total glucosinolates was significantly higher at the rapid growth stage but when biomass is taken into account, highest ITC release potential, and advised time of incorporation, is the same as for the mustard cultivars: when half the crop has flowered.

What is the optimal seeding rate for maximum ITC release potential?

Final ITC release potential is dependent on both field biomass and glucosinolate concentrations which generally varied only slightly between the tested seeding rates. For the mustards *B. juncea* and *S. alba*, lower seeding rates resulted in as much ITC release potential as higher seeding rates. The *B. juncea* cultivars: Vitasso, Scala and ISCI99 seeded at rates ranging from 6-12 kg/ha produced similar yields ranging between 29 and 35 tonnes/ha. Other biofumigation studies have made use of higher biomass ranges for *B. juncea*: 90-115 t/ha *(23)*, 122 t/ha *(31)*, 45 t/ha *(32)*, and for *S. alba*: 91.6 t/ha *(18)*. McKenzie *et al.* (2006) reported that seed yield in *S. alba* and *B. juncea* were affected

by seeding rate only in extremely dry conditions and work carried out on *B. napus* also showed that plant density has very little effect on final seed yield *(33-35)*. In this study, the more sparsely sown mustard plants grew larger, fully compensating for their reduced number by the time they had developed to flowering. For the radish cultivars, higher seeding rates resulted in larger biomass with a doubling of seeding rate from 10 to 20 kg/ha resulting in an average increase in total biomass of ~40% and ~17% for Bento and Diablo respectively. However, the higher proportion of stem for radish cultivars at higher seeding rates may affect ITC release downstream as stems are tougher and harder to mulch. It is likely other factors such as climate, fertiliser and soil conditions are more instrumental than seeding rates in determining final biomass yields.

Best practice for maximising GSL content at incorporation

Growers' goals and circumstances must be considered when assessing the real world applicability of biofumigation. Legume cover crops are reportedly the most reliable means to enhance cash crop yields, yet if soil pests are a major yield limiting factor the use of Brassica cover crops could be considered *(36)*. Prohibitive costs of seed and fertilizer as well as comparative costs and benefits of other cover crops may limit the uptake of biofumigation as a means to tackle soil pests. Recommended seeding rates offered by commercial suppliers of biofumigants vary substantially (from 6 to over 20 kg/ha) and this can have a substantial impact on the final costs. This is the first study which examines the impact of plant density on GLS potential and has demonstrated that lower seeding rates are capable of producing comparable biofumigation potentials. Our data suggest that a seeding rate of 8-10 kg/ha for *B. juncea* (cv. ISCI99) and *S. alba* (cv. Ida Gold) and a seeding rate of 10-15 kg/ha for *R. sativus* (cv. Bento) and incorporation at 50% flowering results in the highest GSL potential. The benefits of green manure are well established, such that the biomass of brassica used in biofumigation may play an important role independent of its glucosinolate concentrations. These benefits include positive impacts on organic matter, nutrition, soil structure and erosion control *(37,*

38). Brassica green manure crops are specifically reported to reduce wind erosion, and prevent nitrogen leaching from the soil *(38)*. The slightly increased biomass in *R. sativus* cultivars grown at higher seeding rates may have field benefits, but valuing them against the additional cost incurred is not possible within the scope of this paper.

Conclusion

Incorporation of brassica cover crops into agricultural soils as part of an integrated pest management system has been associated with a range of benefits including direct pest suppression, reduced soil erosion and reduced nitrogen leaching. In terms of biofumigation benefits, we have demonstrated that changes in seed density have very little effect on the final biomass of the biofumigant and the glucosinolate concentration at the time of incorporation. These findings suggest that farmers may be able to to minimise costs by reducing seeding rate without negatively affecting GSL potential. More work needs to be done to verify this in field studies. Glucosinolate profiles differed only marginally between cultivars of the same species but differed considerably between species. In addition, we have re-affirmed that incorporation of mustards should occur at 50% flowering when glucosinolate concentrations and plant biomass are at their highest.

Materials and methods

Plant material

Brassica juncea (cv. ISCI99, cv. Scala and cv. Vitasso), *R. sativus* (cv. Bento and cv. Diablo), and *S. alba* (cv. Ida Gold) plants were grown by Barworth Agriculture Ltd. in a sandy loam soil dominated field (coordinates: 53.000371, -0.290404). 90kg of nitrogen in the form of ammonium sulphate were added to the field which was subdivided into randomised block plots 1.6m x 12m.*Brassica juncea* (cv. ISCI99) and *S. alba* (cv. Ida Gold) were grown from 07-08-2014 to 25-10-2014. Total stem and total leaves were cut from plants at three growth stages: (i) early rapid growth, (ii) 50 % maturity, and (iii)

50% flowering. These stages corresponded to (i) 25-9-2014 (49 days post drilling), (ii) 10-10-2014 to 14-10-2014 (64-68 days post drilling), (iii) 25-10-2014 (79 days post drilling) for *B. juncea* and *S. alba* and (i) 11-8-2014 (42 days post drilling), (ii) 21-8-2014 (52 days post drilling), (iii) 27-8-2014 (58 days post drilling) for *R. sativus*. Plants were sampled at 4 metres and 8 metres along each plot (1m x 0.5m) to give a combined total sample area of $1m^2$ for each plot, 3-6 biological replicates were sampled for biomass and GLS analysis. Leaves and Stems were weighed and sub samples frozen and stored at -80°C for a maximum of 3 months prior to processing.

Samples wrapped loosely in aluminium foil were transported on dry ice and loaded into a LYOTRAP Scientific Ltd. Freeze drier with a cooling plate. Pressure was reduced to 0.12 mbar. Loading took under 2 minutes and samples were dried for 24 hours.

Freeze dried plant tissue was homogenised using a grinder (Lloytron, E5601BK). Homogenised ground samples were milled at a frequency of 20 /s for 3 minutes (Retch, MM400) with 2 steel ball bearings and then sealed and stored at 20°C for up to 1 year.

Glucosinolate extraction

Extractions were carried out using a method adapted from Herzallah and Holley, 2012 which was found to be as accurate as the more commonly used ISO method for analysing indole and aromatic glucosinolates in these species *(39, 40)*. In a subset of samples 50 µl of a 5 mM gluctropaeolin (for *B. juncea* samples) or 20 mM sinigrin (for all other samples) internal standard was added.

Boiling water extraction:

25 ml of boiling water was added to 0.1 g of freeze dried and milled plant tissue in a 150 ml erlenmeyer flask and the internal standard was added. The sample was incubated at 100°C and stirred on a magnetic hot plate for 10 minutes. The sample was incubated for a further 4 h at 70°C before centrifugation at 4000 rpm (Jouan, model:B 3.11) for 10 minutes. The sample was topped up to 20 ml with deionised water.

HPLC analysis of intact glucosinolates – (adapted from Herzallah and Holley, 2012)

A C18 column (Phenomonex, SphereClone 5u ODS(2)) was equilibrated for 1 h with a mobile phase which consisted of 80% (0.02 M) TBA and 20% ACN with detection at 229 nm. The flow rate was set at 1.0 ml/min and separated according to the program outlined in table 2.

Time	% solution A	% solution B	Transition
0	100	0	
30	0	100	Linear gradient
35	0	100	
40	100	0	Linear gradient
50	100	0	

Table 2: Mobile phase conditions for separation of desulfoglucosinolates.

Solution A: 100% TBA (0.02M)

Solution B: 70:30, TBA (0.02M):acetonitrile

Glucosinolates were quantified using the chromatogram from 229 nm and standard curves were constructed using pure sinigrin (Sigma Aldrich), glucotropaeolin, glucoraphenin, glucoraphanin, glucoerucin, glucobrassicin, gluconasturtiin, sinalbin, progoitrin and glucoiberin (all from Phytoplan).

In the case of glucoraphasatin in *R. sativus* leaves and glucotropaeolin in *B. juncea* minor alterations were made to avoid peaks co-eluting. The mobile phase programme for *R. sativus* leaves was 100% A for 5 minutes, followed by a 35 minute linear gradient to 66% B followed by a 5 minute linear gradient to 100% B followed by a 5 minute linear gradient to 100% A . For *B. juncea* leaves, an isocratic 85:15, TBA (0.02M):acetonitrile mobile phase for 70 minutes was used.

Statistical analyses

For determination of significance of effect of seeding rate, tissue type, and cultivar on final glucosinolate content, plant biomass and glucosinolate field potential, ANOVA analyses were carried out. TukeyHSD post Hoc analyses were carried out to determine significance within groups. Statistical analyses were carried out with R statistical software package (version 3.3.1).

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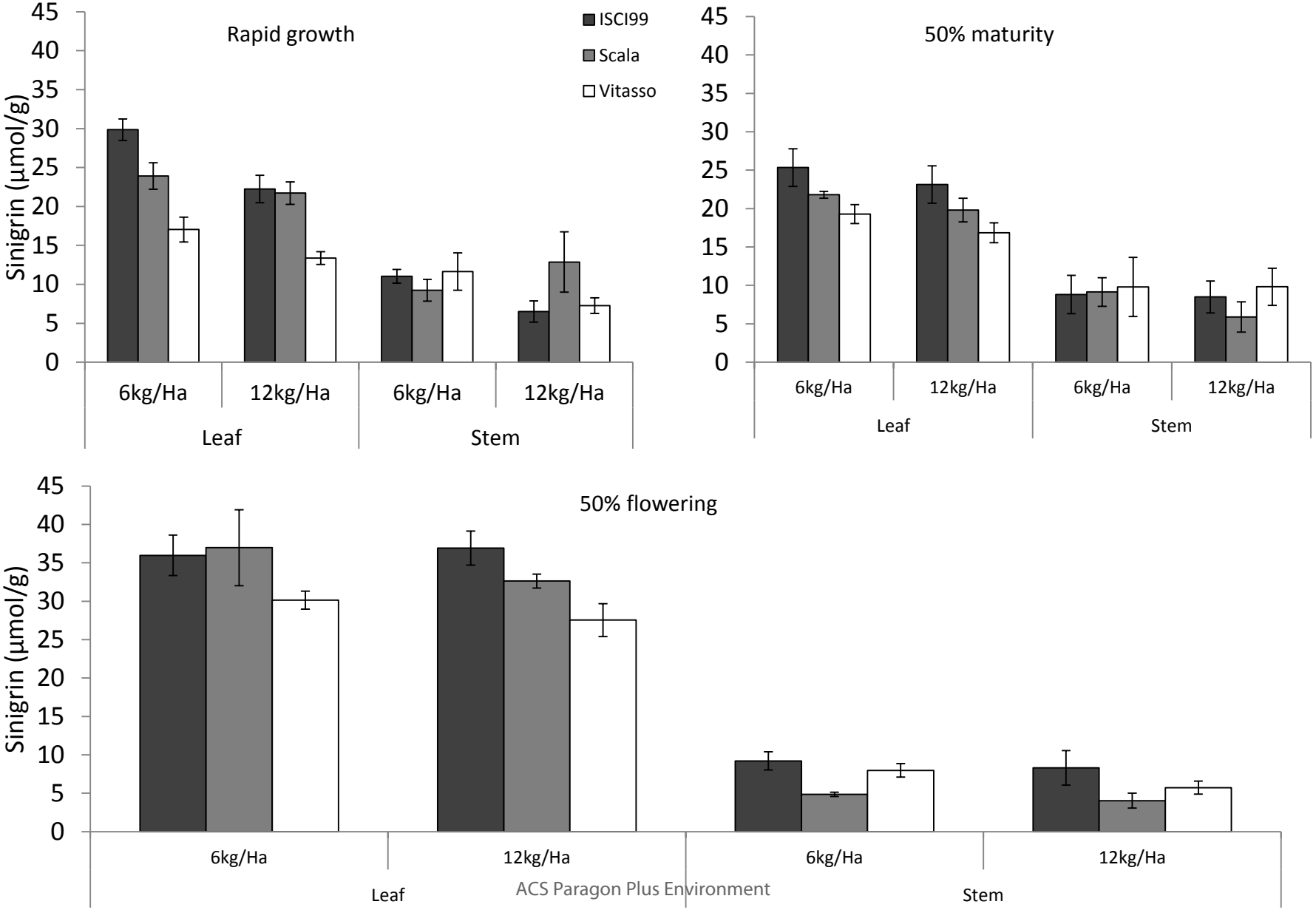
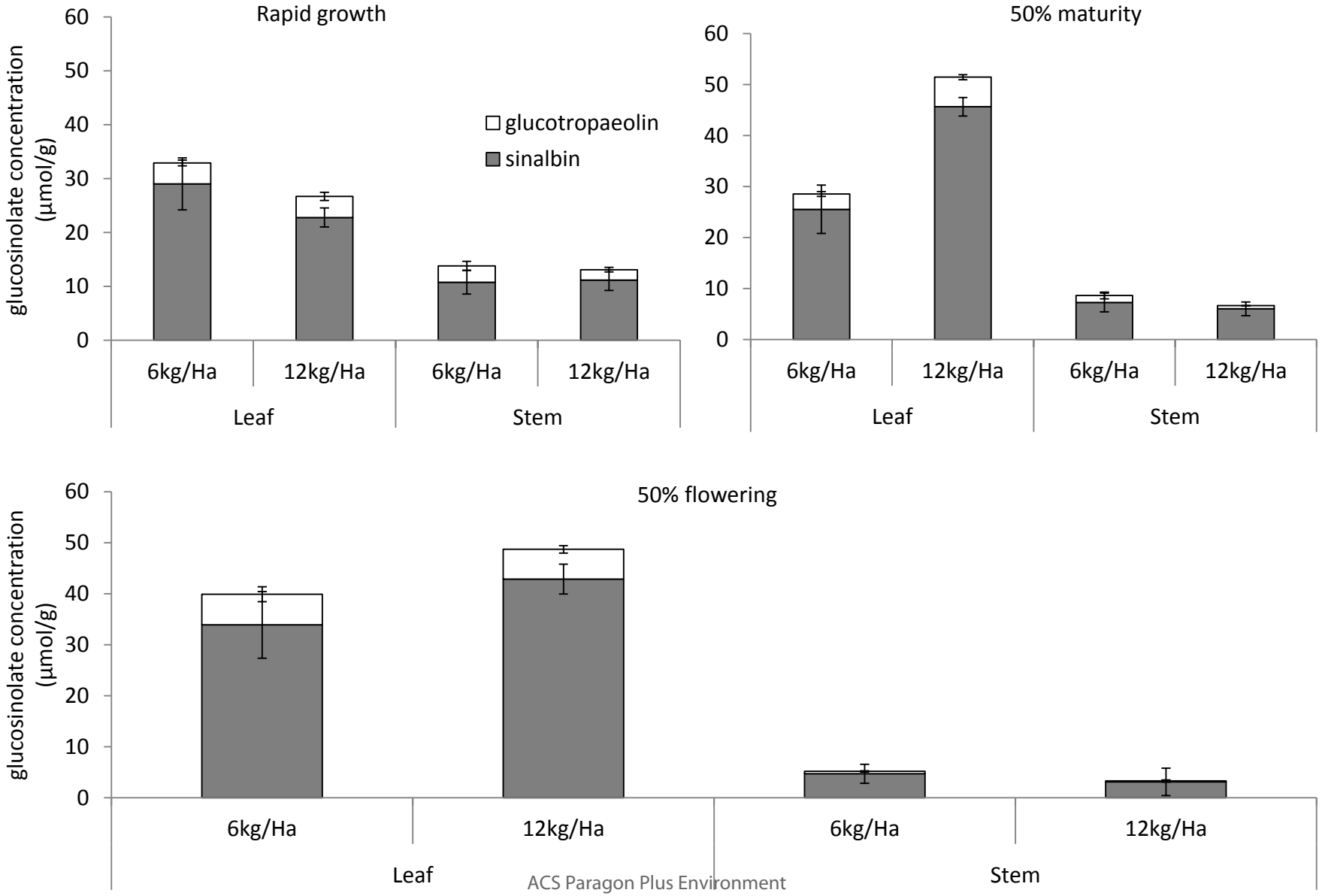
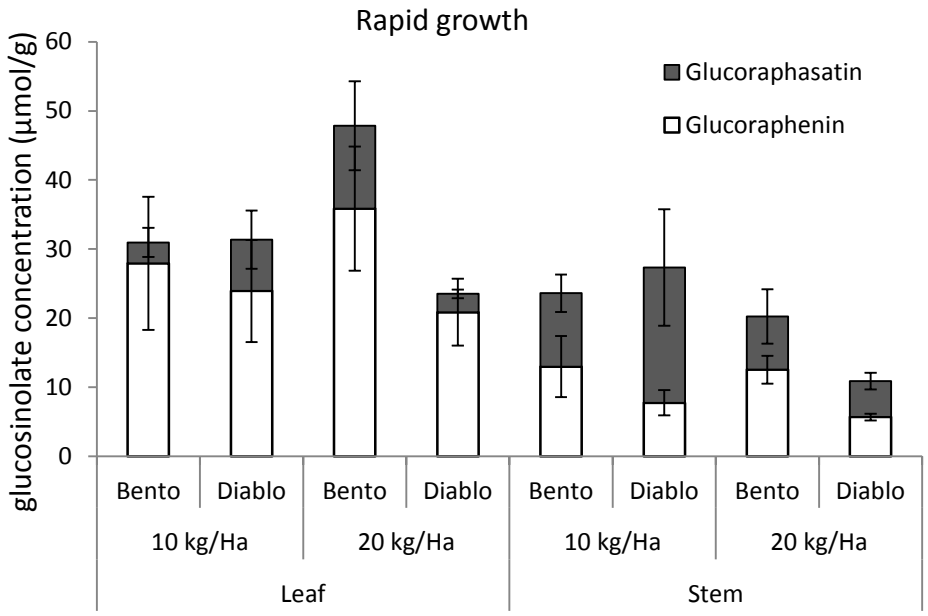
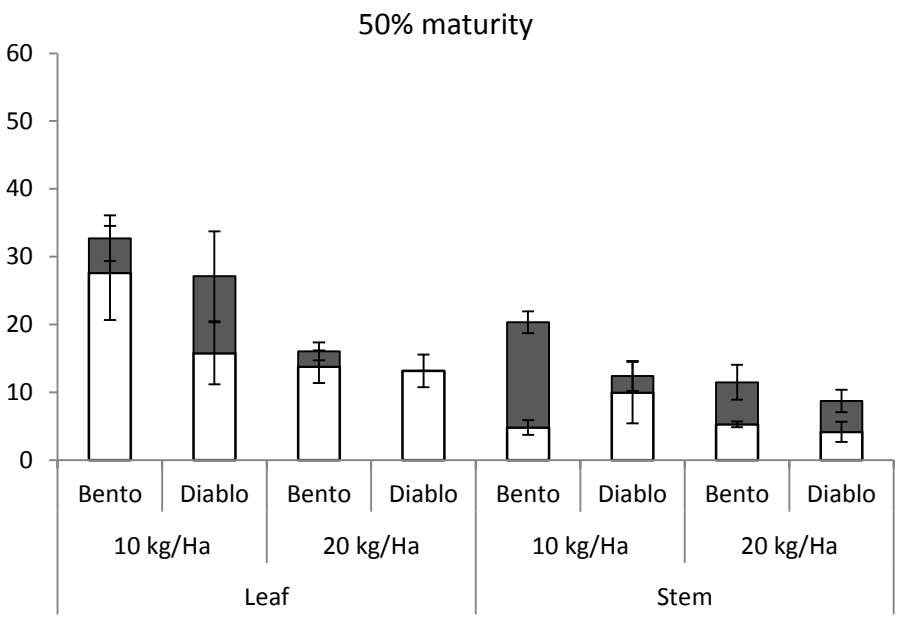

Rapid growth
ISCI99
Scala
Vitasso
Sinigrin (µmol/g)
45
40
35
30
25
20
15
10
5
0
6kg/Ha
12kg/Ha
6kg/Ha
12kg/Ha
Leaf
Stem
50% maturity
6kg/Ha
12kg/Ha
6kg/Ha
12kg/Ha
Leaf
Stem
50% flowering
Sinigrin (µmol/g)
6kg/Ha
12kg/Ha
6kg/Ha
12kg/Ha
Leaf
Stem


Rapid growth
50% maturity
50% flowering
glucosinolate concentration (µmol/g)
glucotropaeolin
sinalbin
0
10
20
30
40
50
60
6kg/Ha
12kg/Ha
Leaf
Stem


Rapid growth
Glucoraphasatin
Glucoraphenin
glucosinolate concentration (µmol/g)
60
50
40
30
20
10
0
Bento
Diablo
Bento
Diablo
Bento
Diablo
Bento
Diablo
10 kg/Ha
20 kg/Ha
10 kg/Ha
20 kg/Ha
Leaf
Stem


50% maturity
60
50
40
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10 kg/Ha
20 kg/Ha
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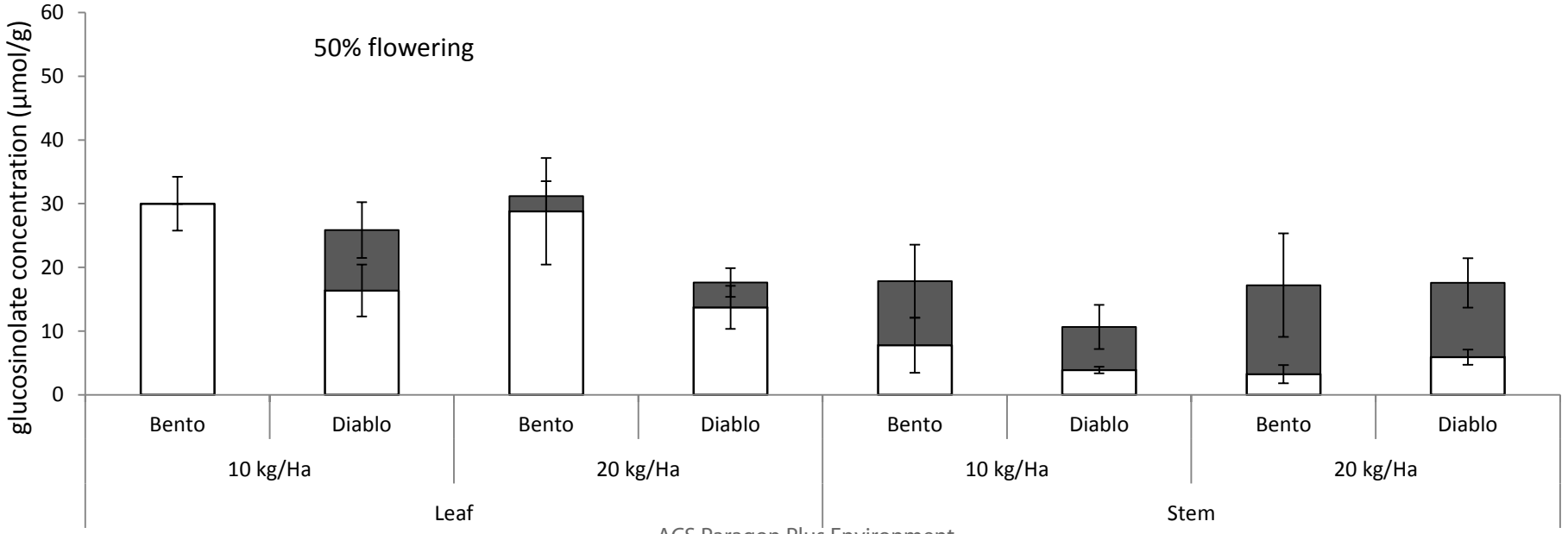
20 kg/Ha
Leaf
Stem


50% flowering
glucosinolate concentration (µmol/g)
60
50
40
30
20
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Bento
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Diablo
10 kg/Ha
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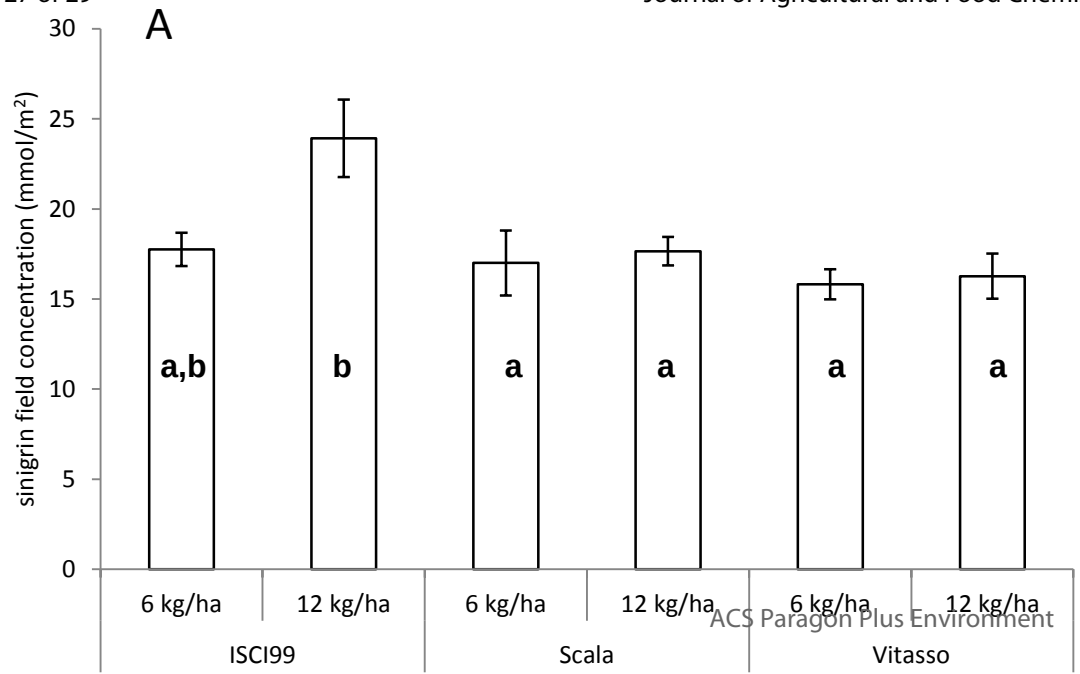
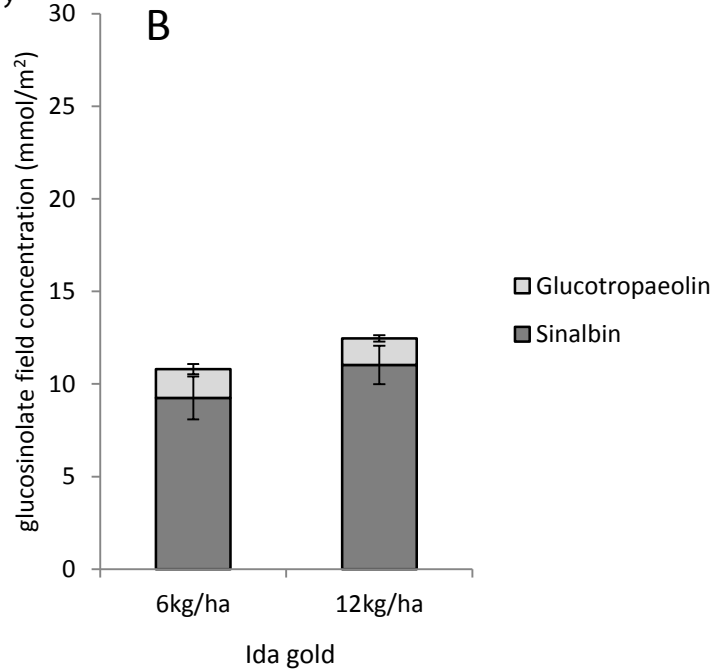
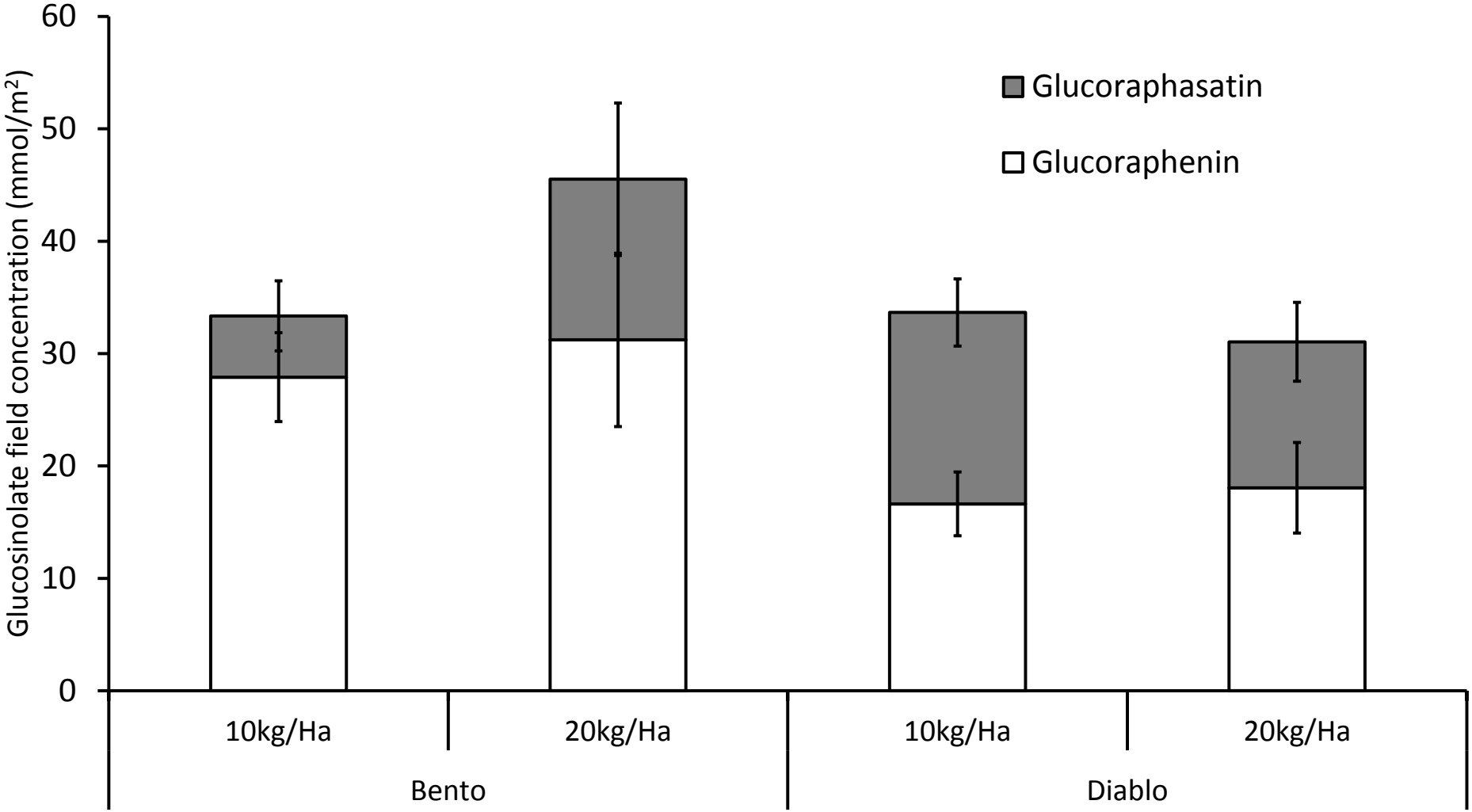
Leaf
Stem






60
50
40
30
20
10
0
Glucosinolate field concentration (mmol/m²)
Glucoraphasatin
Glucoraphenin
10kg/Ha
20kg/Ha
10kg/Ha
20kg/Ha
Bento
Diablo