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**Potential isothiocyanate release remains constant across
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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.

20 Introduction

21

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

34 Quantification of isothiocyanates is analytically challenging and therefore glucosinolate
35 concentrations in plant tissues have been used as a proxy to estimate potential isothiocyanate
36 release in the field. Kirkegaard and Sarwar (1998) examined variation in biomass and glucosinolate
37 profiles of 80 different brassicas to explore their possible use as biofumigants and found significant
38 variation in GSL field potential (i.e. the concentration of glucosinolates per field area) ranging from
39 0.8 to 45.3 mmol m⁻² (7). GSL field potentials alone may be misleading however, since glucosinolate
40 profiles (the types and relative amount of glucosinolates produced) vary between species and
41 determine the type and quantity of ITC release which determines the overall biofumigation effect
42 (1). For instance, *Brassica napus* mainly produces indole glucosinolates which do not form ITCs,
43 while other species, such as *Brassica juncea* and *Sinapis alba*, predominantly produce aliphatic and
44 aromatic glucosinolates respectively (7). Additionally, biofumigant selection must also take into

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

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

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

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

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

- 88 (1) Which cultivar has maximum GSL release potential among a sub-set of commercial cultivars?
- 89 (2) Can we confirm that maximum field GSL content is reached at the 50% flowering stage.
- 90 (3) What is the optimal seeding rate for maximum ITC release potential?

91 **Results**

92 **Effect of seeding rate on biofumigant biomass**

93

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

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

119

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

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

123

124 Glucosinolate concentration

125

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

128 *sativus*) at three growth stages: rapid growth, 50% maturity, and 50% flowering. Sinigrin made up
129 more than 90% of total glucosinolate content in green tissues of *B. juncea* cultivars with higher
130 concentrations found in the leaves (fig 1). There was a significant effect of cultivar and a significant
131 combined effect of cultivar and tissue type on sinigrin concentration: cultivars differed significantly
132 in leaf sinigrin concentrations but not in stem sinigrin concentrations (supplementary tables 2.1 to
133 2.2). Sinigrin concentrations in ISCI99 leaves were on average ~12% and ~29% higher than in Scala
134 and Vitasso leaves respectively. There was a significant effect of seeding rate on sinigrin
135 concentrations which were on average 20% higher at the lower rate of 6kg/ha (fig 1, supplementary
136 table 2.1). Sinigrin concentrations were modulated by growth stage with mean concentration in
137 leaves significantly higher at 50% flowering relative to both other growth stages and mean
138 concentration in the stems significantly lower at 50% flowering relative to the rapid growth stage (fig
139 1, supplementary table 2.3).

140 **FIGURE 1**

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

144 *S. alba* (Ida Gold) does not produce the aliphatic glucosinolate sinigrin in appreciable amounts.
145 Glucotropaeolin and sinalbin are both aromatic glucosinolates and accounted for over 90% of the
146 total glucosinolate content in the green biomass of *S. alba* (cv. Ida gold) (fig 2). A significant
147 statistical three way interaction was observed between the effects of tissue type, seeding rate and
148 growth stage on total glucosinolate concentration (ANOVA: $F_{(2, 32)}=5.22$; $p=0.011$). Total
149 glucosinolate concentration was significantly higher in leaves in all conditions (fig 2, supplementary
150 tables 2.2 to 2.2.4). Leaf glucosinolate concentration was higher at the 50% flowering stage (~45
151 $\mu\text{mol.g}^{-1}$) than the rapid growth stage (~30 $\mu\text{mol.g}^{-1}$) and stem glucosinolate concentration
152 decreased with plant growth stage (from approximately 12 $\mu\text{mol.g}^{-1}$ at rapid growth to 5 $\mu\text{mol.g}^{-1}$ at

153 50% flowering) (fig 2, supplementary table 2.2.1). On average, the differences and relative
154 contributions of glucosinolate concentrations in the leaf and stem increased over time.
155 Glucosinolate concentrations were significantly higher at higher seeding rates only for leaves
156 sampled from plants at 50% maturity (supplementary table 2.2.3).

157 **FIGURE 2**

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

161 Glucoraphenin and glucoraphasatin are both aliphatic glucosinolates and account for over 90% of
162 ITC releasing glucosinolates in *R. sativus* (cv. Diablo and cv. Bento) shoots. Variability of
163 glucosinolates within sample sets was much higher than with the mustards (fig 3). Concentrations of
164 glucoraphenin were significantly higher in Bento than in Diablo, and in leaves than in stems (ANOVA:
165 $F_{(1, 64)} = 9.143$; $p < 0.01$; and ANOVA: $F_{(1, 64)} = 54.164$; $p < 0.001$ respectively)(supplementary table 2.3.1).
166 A significant effect of growth stage on glucoraphenin was also identified (ANOVA: $F_{(2, 64)} = 3.521$;
167 $p = 0.035$). There was a three way interactive effect of growth stage, tissue type and seeding rate on
168 glucoraphasatin concentrations (ANOVA: $F_{(2, 64)} = 3.823$; $p = 0.027$) that were significantly lower in leaves
169 sampled at 50% maturity from plants at 20kg/ha relative to stems sampled at rapid growth at
170 10kg/ha (supplementary tables 2.3.2 and 2.3.2.1). No interactive effect of any combination of
171 seeding rate, growth stage, cultivar, and tissue type on glucoraphenin concentration was detected.
172 Total glucosinolate concentrations were significantly higher in Bento than in Diablo and in leaves
173 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
174 significant effect of growth stage on total glucosinolate concentrations was also identified
175 (ANOVA: $F_{(2, 62)} = 4.143$, $p = 0.020$)(Supplementary table 2.3.3). The glucosinolate concentrations from
176 radish plants sampled at the rapid growth stage were significantly higher than total glucosinolate
177 concentrations from radish plants sampled at the 50% maturity stage (TukeyHSD, $p_{adj} = 0.016$). No

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

180 **FIGURE 3**

181

182 Figure 3: Glucoraphasatin and glucoraphanin concentrations in field grown leaves and stems of *R. sativus* (cv.
183 Bento and cv. Diablo) sampled during rapid growth, at 50% maturity and at 50% flowering. Error bars
184 represent standard error (n=3-4). Results from statistical analyses can be found in supplementary tables 2.3 to
185 2.3.3.3.

186

187 **Glucosinolate concentration in the field.**

188

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

202 growing Ida Gold at a seeding rate of 6kg/ha than in fields growing the *B. juncea* cultivars
203 (supplementary table 3.2.2). Diablo (*R. sativus*) mean glucosinolate concentrations ranged from 13
204 to 17 mmol/m² and 16 to 18 mmol/m² for glucoraphasatin and glucoraphenin respectively (fig 5).
205 Bento (*R. sativus*) mean glucosinolate concentrations ranged from 5.4 to 14 mmol/m² and 28 to 31
206 mmol/m² for glucoraphasatin and glucoraphenin respectively. No significant difference in total
207 glucosinolate concentrations was identified between the cultivars or seeding rates for *R. sativus*, but
208 concentrations of glucoraphenin were significantly higher in Bento than in Diablo (ANOVA:F_(1, 11)=
209 5.316; p=0.042)(supplementary table 3.4).

210

211 **FIGURE 4**

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

216 **FIGURE 5**

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

220 **Discussion**

221

222 **Which commercial biofumigant cultivar has the highest ITC release** 223 **potential?**

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

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

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

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

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

254 *B. juncea* ISCI99 fields produced higher glucosinolate concentrations (24 mmol.m⁻² glucosinolate at a
255 drilling rate of 20 kg/ha and at 50% flowering) than *B. juncea* Scala and *B. juncea* Vitasso (~17
256 mmol.m⁻² and ~16 mmol.m⁻² respectively). Incorporation of *B. juncea* in field trials is reported to
257 control plant parasitic nematode species including: *Tylenchus semipenetrans* (21), *Meloidogyne*
258 *chitwoodi* (22), *Meloidogyne javanica* (23), *Globodera pallida* (16), and fungal species: *Sclerotinia*
259 *minor* (21), *Rhizoctonia solani* (24). However, other studies have reported no effect of *B. juncea*
260 incorporation on some of these same species (25, 4, and 1). As a result of this variability in efficacy,
261 the major glucosinolate found in *B. juncea* cultivars, namely sinigrin, has been the subject of many
262 studies relating to biofumigation. Allyl isothiocyanate (AITC), a product of sinigrin hydrolysis, is often
263 reported to be one of the most toxic naturally occurring isothiocyanates by virtue of its short side
264 chain and high volatility (1). Sarwar et al. (1998) reported that AITC and methyl isothiocyanate were
265 similarly effective in their ability to suppress mycelial growth of five root pathogens in vapour
266 exposure headspace experiments (20). Depending on the plant and type of control required, an
267 estimated 517 to 1294 nmol/g soil of methyl ITC is required for soil sterilisation (26). Our results
268 indicate a maximum AITC potential of 16-24 mmol/m² which, assuming a soil bulk density of 1.4 g
269 cm⁻³ and incorporation to 20 cm, would give a considerably lower maximum of 85 nmol/g. In
270 addition, given that ITC production is dependent on soil conditions, incorporation depths can be
271 substantially deeper, and glucosinolates are unlikely to be fully converted to ITC, true ITC
272 concentrations following incorporation are likely to be even lower (27). In addition, methyl ITC is
273 reported to have higher activity than AITC in vapour exposure experiments and in soil experiments
274 (15). While initial AITC release at these levels is not enough to completely sterilise the soil, soil
275 pathogen control observed in numerous biofumigation studies may result from a cocktail of toxic
276 chemicals (including DMS) to which AITC concentrations contribute (6).

277 **When should biofumigants be incorporated for maximum ITC release**
278 **potential?**

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

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

295 Final ITC release potential is dependent on both field biomass and glucosinolate concentrations
296 which generally varied only slightly between the tested seeding rates. For the mustards *B. juncea*
297 and *S. alba*, lower seeding rates resulted in as much ITC release potential as higher seeding rates.
298 The *B. juncea* cultivars: Vitasso, Scala and ISCI99 seeded at rates ranging from 6-12 kg/ha produced
299 similar yields ranging between 29 and 35 tonnes/ha. Other biofumigation studies have made use of
300 higher biomass ranges for *B. juncea*: 90-115 t/ha (23), 122 t/ha (31), 45 t/ha (32), and for *S. alba*:
301 91.6 t/ha (18). McKenzie *et al.* (2006) reported that seed yield in *S. alba* and *B. juncea* were affected

302 by seeding rate only in extremely dry conditions and work carried out on *B. napus* also showed that
303 plant density has very little effect on final seed yield (33-35). In this study, the more sparsely sown
304 mustard plants grew larger, fully compensating for their reduced number by the time they had
305 developed to flowering. For the radish cultivars, higher seeding rates resulted in larger biomass with
306 a doubling of seeding rate from 10 to 20 kg/ha resulting in an average increase in total biomass of
307 ~40% and ~17% for Bento and Diablo respectively. However, the higher proportion of stem for
308 radish cultivars at higher seeding rates may affect ITC release downstream as stems are tougher and
309 harder to mulch. It is likely other factors such as climate, fertiliser and soil conditions are more
310 instrumental than seeding rates in determining final biomass yields.

311

312 **Best practice for maximising GSL content at incorporation**

313 Growers' goals and circumstances must be considered when assessing the real world applicability of
314 biofumigation. Legume cover crops are reportedly the most reliable means to enhance cash crop
315 yields, yet if soil pests are a major yield limiting factor the use of Brassica cover crops could be
316 considered (36). Prohibitive costs of seed and fertilizer as well as comparative costs and benefits of
317 other cover crops may limit the uptake of biofumigation as a means to tackle soil pests.
318 Recommended seeding rates offered by commercial suppliers of biofumigants vary substantially
319 (from 6 to over 20 kg/ha) and this can have a substantial impact on the final costs. This is the first
320 study which examines the impact of plant density on GLS potential and has demonstrated that lower
321 seeding rates are capable of producing comparable biofumigation potentials. Our data suggest that a
322 seeding rate of 8-10 kg/ha for *B. juncea* (cv. ISCI99) and *S. alba* (cv. Ida Gold) and a seeding rate of
323 10-15 kg/ha for *R. sativus* (cv. Bento) and incorporation at 50% flowering results in the highest GSL
324 potential. The benefits of green manure are well established, such that the biomass of brassica used
325 in biofumigation may play an important role independent of its glucosinolate concentrations. These
326 benefits include positive impacts on organic matter, nutrition, soil structure and erosion control (37,

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

331 Conclusion

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

342 Materials and methods

343

344 Plant material

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

351 50% flowering. These stages corresponded to (i) 25-9-2014 (49 days post drilling), (ii) 10-10-2014 to
352 14-10-2014 (64-68 days post drilling), (iii) 25-10-2014 (79 days post drilling) for *B. juncea* and *S. alba*
353 and (i) 11-8-2014 (42 days post drilling), (ii) 21-8-2014 (52 days post drilling), (iii) 27-8-2014 (58 days
354 post drilling) for *R. sativus*. Plants were sampled at 4 metres and 8 metres along each plot (1m x
355 0.5m) to give a combined total sample area of 1m² for each plot, 3-6 biological replicates were
356 sampled for biomass and GLS analysis. Leaves and Stems were weighed and sub samples frozen and
357 stored at -80°C for a maximum of 3 months prior to processing.

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

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

364 **Glucosinolate extraction**

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

369 **Boiling water extraction:**

370 25 ml of boiling water was added to 0.1 g of freeze dried and milled plant tissue in a 150 ml
371 erlenmeyer flask and the internal standard was added. The sample was incubated at 100°C and
372 stirred on a magnetic hot plate for 10 minutes. The sample was incubated for a further 4 h at 70°C
373 before centrifugation at 4000 rpm (Jouan, model:B 3.11) for 10 minutes. The sample was topped up
374 to 20 ml with deionised water.

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

377 A C18 column (Phenomenex, SphereClone 5u ODS(2)) was equilibrated for 1 h with a mobile phase
 378 which consisted of 80% (0.02 M) TBA and 20% ACN with detection at 229 nm. The flow rate was set
 379 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	

380 Table 2: Mobile phase conditions for separation of desulfoglucosinolates.

381 Solution A: 100% TBA (0.02M)

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

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

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

391

392 **Statistical analyses**

393

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

398

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400

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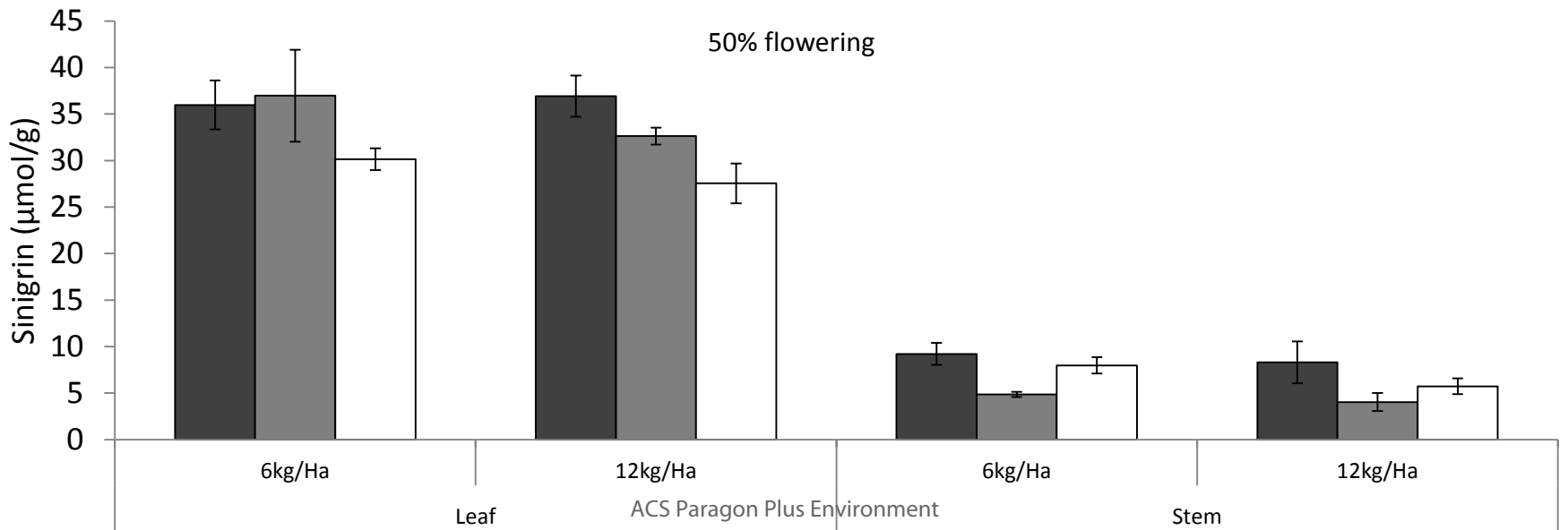
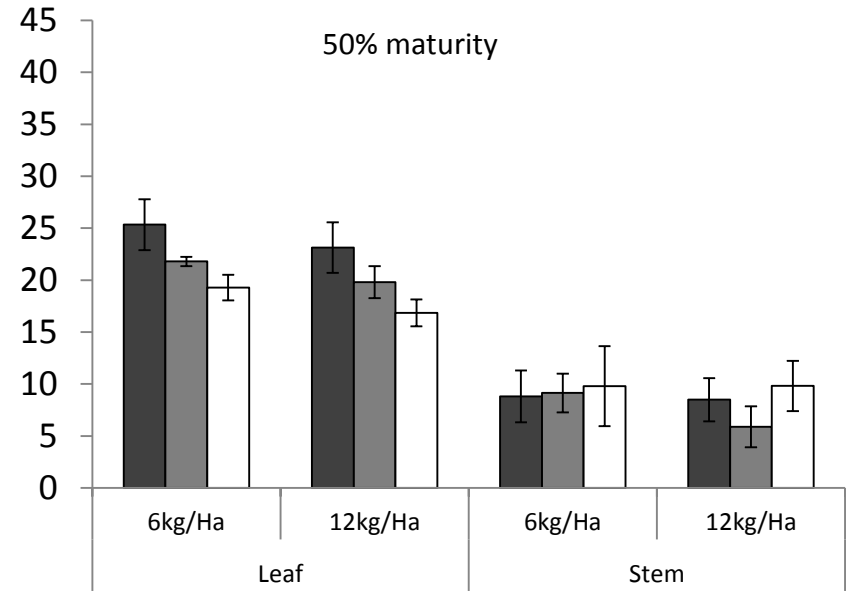
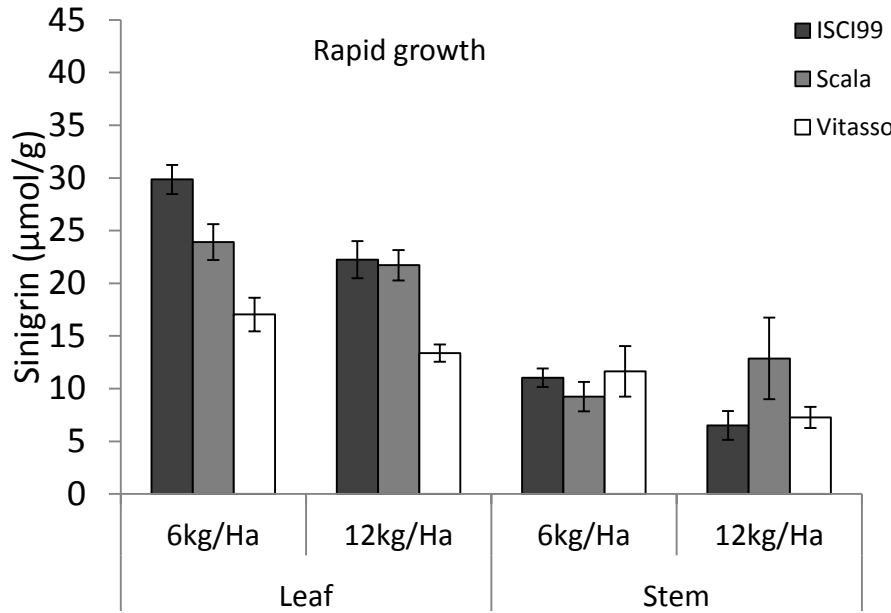
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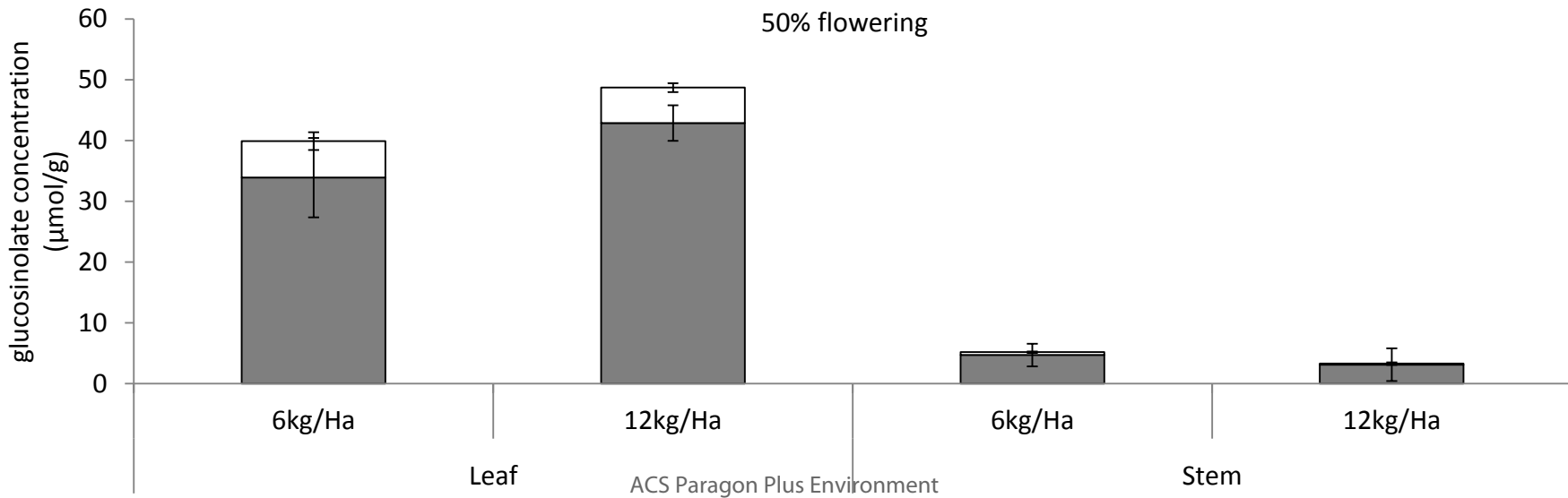
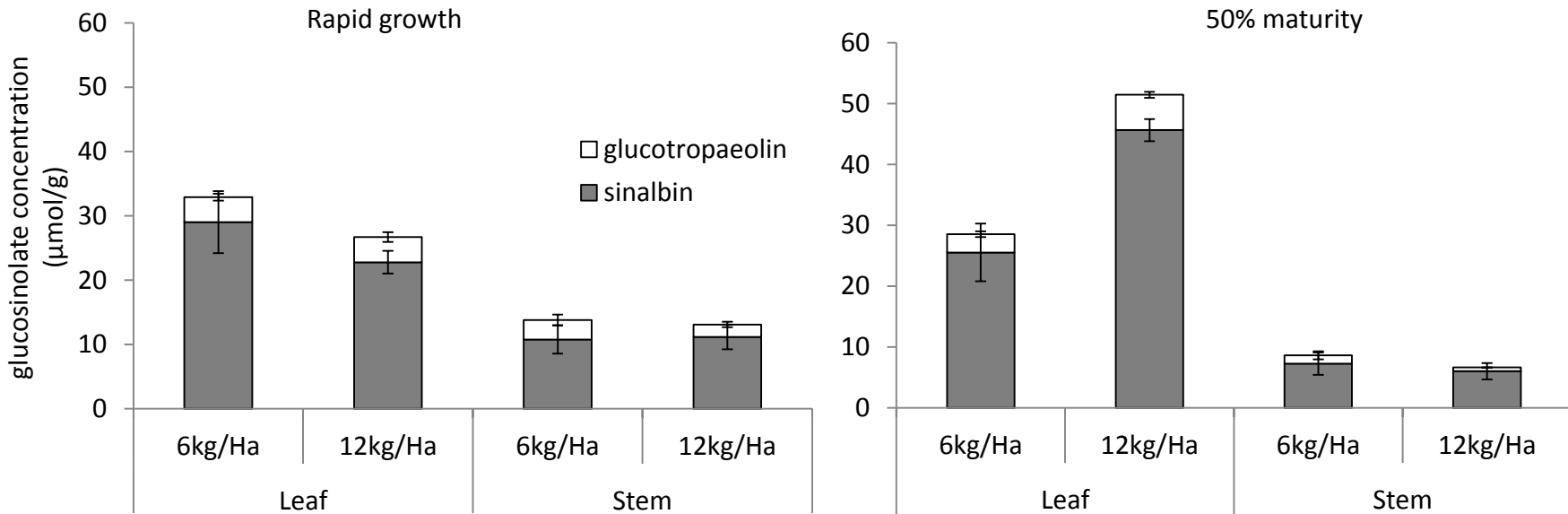
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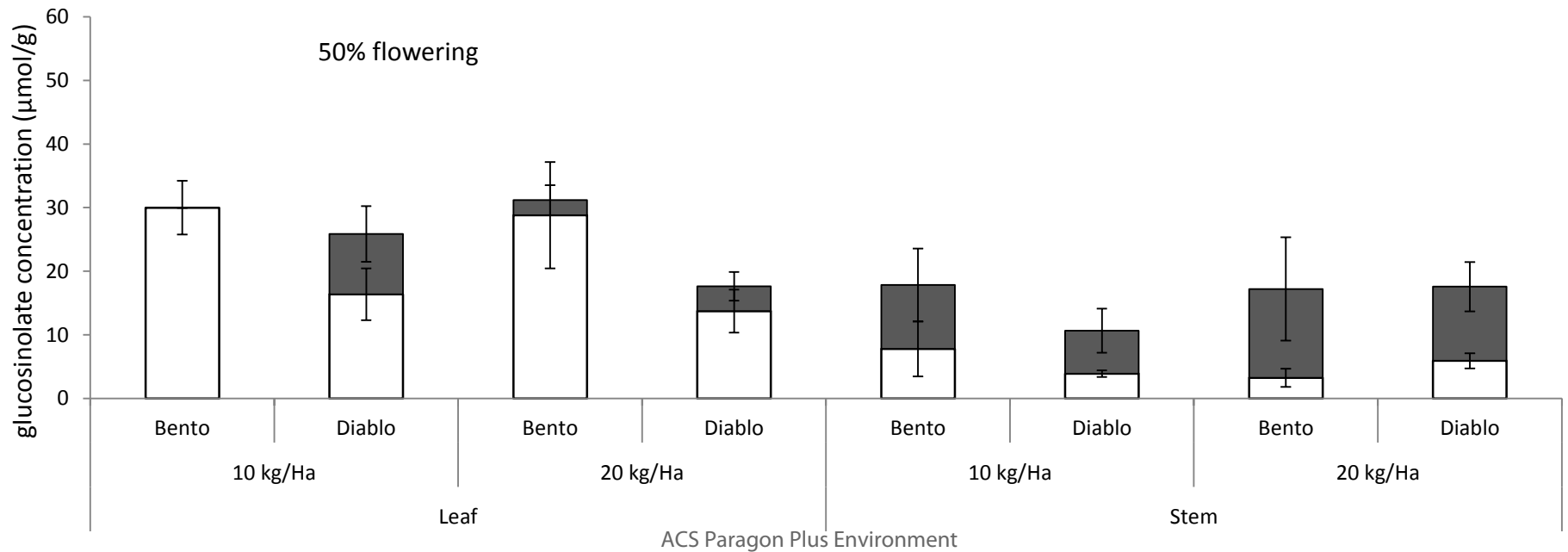
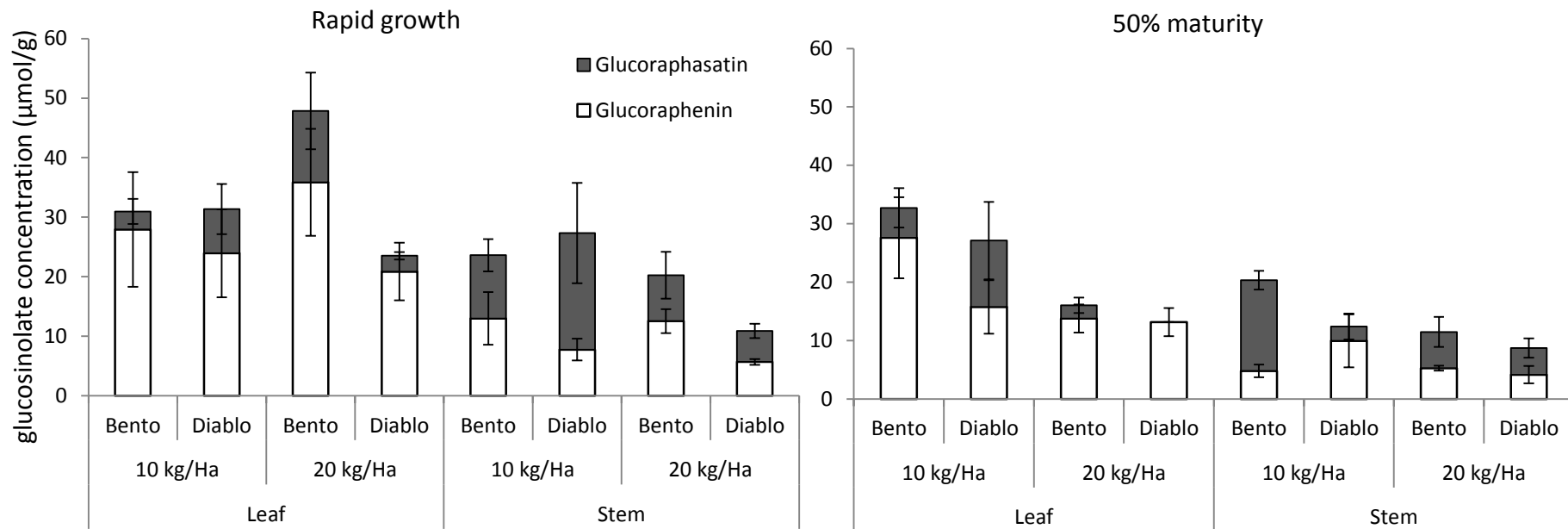
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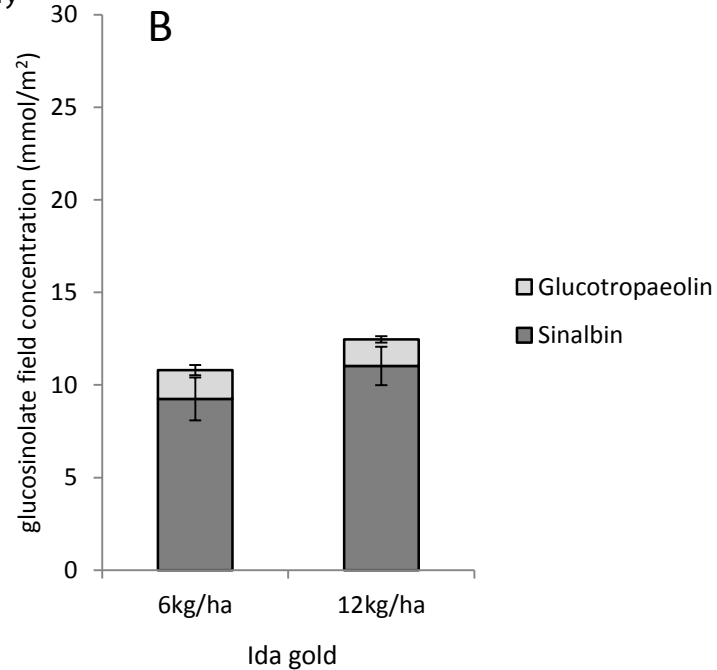
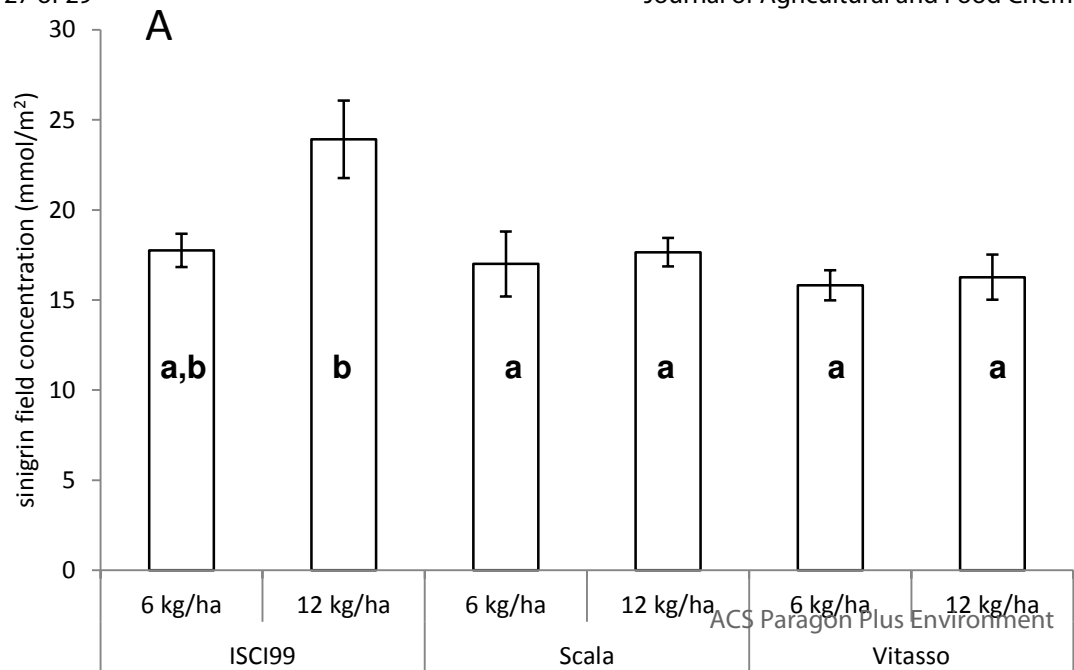
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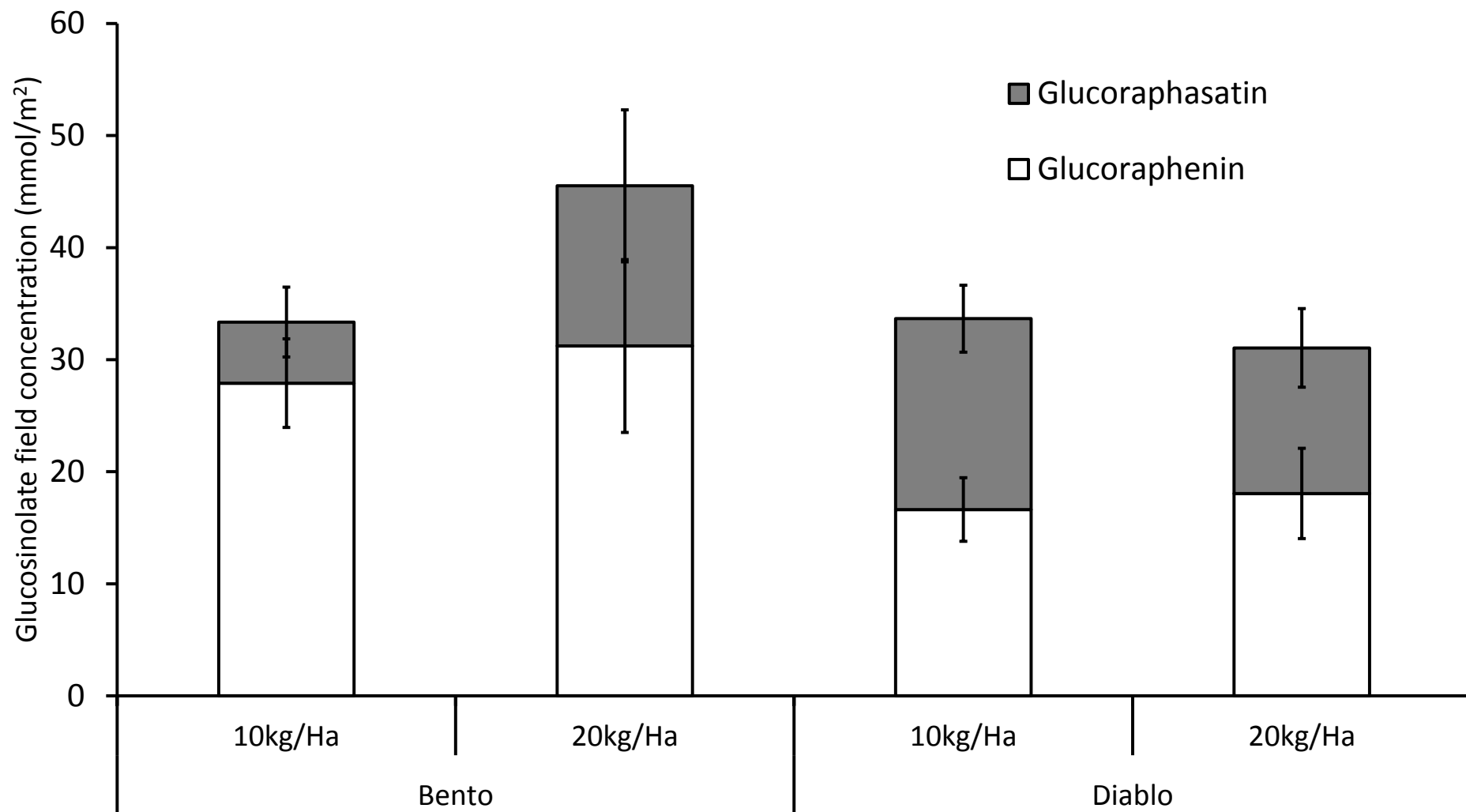
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glucosinolate concentration in field
(mmol/m²)

6 kg/ha ACS Paragon Plus Environment 12 kg/ha
mustard seeding rate

