1 # Maximizing Blad-containing oligomerfungicidal activity in sweet cultivars of *Lupinus albus* seeds

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6

7 **ABSTRACT**

8 During seed germination and plantlet growth, an important aspect of *Lupinus* β-conglutin proteolysis is the accumulation of blad in the
9 cotyledons. Blad, a 20.4 kDa, 173 residue polypeptide which inhibits fungal growth, is the main subunit of BCO (Blad-containing
10 oligomer), a *Lupinu*s bioactive polypeptide oligomer, which underwent a successful translational research during the last thirty years. The
11 development of this recent broad-spectrum biological fungicide for plant disease control made evident the advantage of being non-toxic to
12 the environment, plants, humans and other animals, an interesting characteristic given the increasing consumer's concern about food
13 safety. The industrial-scale production of the edible fungicide BCO for agricultural purposes involves germinating and growing for ca. 8
14 days massive amounts of *Lupinus* plantlets. Therefore, it becomes economically relevant to maximize/increment the amount of
15 extractable BCO. Although BCO represents an alternative pathway for lupin production in Europe, there is no previous evidence on lupin
16 cultivars concerning BCO activity or its cotyledonary concentration, and therefore on the most promising cultivars for BCO extraction
17 and fungicide production. In this work, the amount of BCO and its level of fungicide activity was evaluated in seven sweet cultivars of *L.*
18 *albus* (cvs. Amiga, Energy, Estoril, Ludic, Misak, Multitalia and Rumbo), along 20 days after the onset of germination. In addition, four
19 distinct lots of cv. Energy and six lots of cv Misak, with different harvest years (1997, 2010, 2013 and 2014) and/or different sites of
20 production in Portugal (Alto Alentejo, Baixo Alentejo and Beira Litoral) were also analysed. Quantitative assays demonstrated maximal
21 accumulation of BCO in the cotyledons of 4-days-old *L. albus* plantlets, apart from significant differences between seeds' harvest year or
22 production site. The assays also showed that cv. Energy had significant higher accumulation of BCO (31.67 μg mg^{-1} wet weight) than
23 Rumbo (25.67 μg mg^{-1}) and Misak (22.12 μg mg^{-1}), respectively second and third cultivars with highest accumulation of the oligomer.
24 Additionally, very significant differences were also observed among seeds' harvest year or production site. These observations reduced

25 dramatically the original purpose of this work on the study of BCO variations at the level of sweet L. *albus* cultivars, since the 'noise'
26 detected in BCO levels caused by the year and location of their production was far greater than its variation among cultivars. The
27 observation that storing for several years the seeds at -20ºC did not seem to decrease BCO activity and suggests that the variation in
28 cotyledonary BCO accumulation is essentially controlled by the prevailing edaphoclimatic conditions during seed formation.
29 Furthermore, the activity tests exhibited significant antifungal activity between 3 and 5 days-old plantlets, with absence of antifungal
30 activity on 8 days-old plantlets or older. Overall this study is of considerable importance to maximize BCO extraction from the cotyledons
31 of sweet L. *albus* cultivars.

32 *Keywords*: Blad, BCO, *Lupinus albus*, antifungal activity, fungicide production, seed germination

33

34 **1. Introduction**

35 Climate change may severely influence the effects of plant diseases and pests on crop production, through altered spread of some
36 species and introduction of new pathogens and vectors, leading to uncertain dynamics of plant epidemics (Garrett et al., 2006). CO_2
37 enrichment, N deposition, and changes in temperature and rainfall regimes increase the infection patterns of fungal plant pathogens
38 (Tylianakis et al., 2008). This could potentially lead to an increase in the number of infection events that in turn could determine an
39 increment in the application of agrochemicals (Hannukkala et al., 2007). Additionally, to feed the over 7 billion people that currently
40 inhabit the planet it does not seem to have any practical option but to use massive applications of chemical, toxic fungicides. Such
41 applications result in several negative effects, including development of resistance among the target microorganisms, toxicity to humans,
42 animals and other nontarget organisms, and long environmental retention periods leading to residual toxicities and environmental
43 pollution (Paster and Barkai-Golan, 2008; Raja, 2014; Gakuubi et al., 2017). In an attempt to reduce the toxicity of synthetic fungicides,
44 increased research was dedicated to the search for alternatives to the toxic fungicides for management of pathogenic fungi. The search for
45 compounds of biological origin was at the forefront. Phytochemicals have been recognized as some of the most promising compounds for
46 the development of novel and ecofriendly phytofungicides (Reddy et al., 2007; Anjorin et al., 2013). The primary advantages of using
47 plant-derived antimicrobials in comparison to synthetic chemicals are their lower mammalian toxicity, higher degradability, multiple
48 mechanisms of action, and fewer incidences of the numerous side effects often associated with synthetic chemicals (Raja, 2014). On the
49 other hand, biological fungicides, such as the bacterium *Bacillus subtilis* or the fungus *Trichoderma* spp, may imbalance the microbiota

50 equilibrium typical of many natural environments and frequently need to be re-applied more often than conventional fungicides. Ideally
51 the discovery and development of nontoxic fungicides could provide a valid alternative.
52 BCO (for Blad-Containing Oligomer), an example of translational research undertaken since its discovery in 1991, is an edible
53 210 kDa polypeptide oligomer composed of a mixture of β-conglutin fragments, with blad (20.4 kDa) as its major subunit. β-Conglutin is
54 a globulin and the major storage protein from *Lupinus* seeds (Melo et al., 1994; Ferreira et al., 1999), whereas blad, BCO bioactive
55 subunit, is a 173 amino acid residue polypeptide which comprises residues 109 to 281 of the precursor of β-conglutin (i.e. pro-β-
56 conglutin) (Monteiro et al., 2010). Under natural conditions, BCO accumulates in the cotyledons of *Lupinus* seedlings between the 4th and
57 14th day after the onset of germination, as a stable breakdown product of β-conglutin catabolism (Ramos et al., 1997).
58 Blad exhibits lectin activity (Ramos et al., 1997; Ferreira et al., 2003) and also the catalytic activities of β-*N*-acetyl-**D**-
59 glucosaminidase and of chitosanase (Monteiro et al., 2015). BCO is non-toxic to plants and animals, humans included, an interesting
60 feature given the consumer's concern about food safety. It exhibits a potent, broad spectrum fungicide activity against all fungal species
61 tested, including human, animal and plant pathogens, food spoiling and food poisoning fungi. BCO also shows a strong plant growth
62 biostimulant activity and a weak bactericide activity towards Gram- bacteria.
63 Blad bioactivity survives very high temperatures and extreme pH values, exposure to organic solvents and detergents. It is
64 particularly sensitive to proteolytic attack and to any other condition that leads to peptide bond cleavage (Monteiro et al., 2010). BCO
65 finds application under real, open air agriculture, withstanding the effect of UV sunlight and producing results which equal or exceed
66 those of the commercially available chemical pesticides. Its mechanism of action is multitarget (Pinheiro et al., 2017), suggesting a low
67 probability for the development of resistance mechanisms by the target pathogens. It is included in the FRAC (Fungicide Resistance
68 Action Committee) Code List©2020_Fungicides sorted by mode of action as a Biological with multiple modes of action, with the BM01
69 FRAC code, and has been certified in the US by OMRI (Organic Materials Review Institute; product number cev-10083) for use in
70 organic farming. Several blad applications are protect by four patent families, PCT International Patent Application nos.
71 PCT/IB2006/052403, PCT/EP2011/067824, PCT/EP2011/067821 and PCT/EP2011/067828 (Ferreira et al., 2006; Carreira et al., 2011a,
72 2011b, 2011c).
73 BCO for agricultural application is currently produced at an industrial plant in Cantanhede, Portugal, from *Lupinus albus* sweet
74 cultivars, to eliminate the presence of toxic levels of alkaloids in the final fungicidal formulation. It is now being sold for agricultural

75 purposes in a number of countries and is expected to hit the European market by late 2021. Countries in which BCO is currently on sale
76 and those which are soon expected to commercialize BCO are given at http://www.cev.com.pt/en/markets/. The development of BCO to
77 treat human fungal infections has certainly a huge potential (e.g. *Malassezia* spp.) (Pinheiro et al., 2016; Carreira et al., 2018).

78 Detailed structural studies performed over the last years on both BCO and blad, as well as attempts to produce recombinant blad
79 and a number carefully selected blad variants led to the conclusion that it will be extremely difficult if not impossible, to produce
80 recombinant BCO and/or blad in sufficient amount at a feasible price. Its production will therefore rely on the massive germination and
81 plantlet growth for ca. 8 days of sweet *Lupinus* cultivar seeds, followed by an appropriate extraction methodology.
82 Incrementing/maximising the amount of extractable BCO would therefore be highly relevant and come as a first priority for the BCO
83 industry.

84 Although BCO represents an alternative way for fungal control in agricultural crops, and in particular in those climates and
85 environments (e.g. rich, intensive crops cultivated under greenhouse conditions) which are highly favourable for fungal growth, and may
86 increase industrial interest on lupin production, up to now there is no evidence about the most promising sweet cultivars of *L. albus* for
87 BCO extraction and fungicide production. Also the edaphoclimatic conditions that prevail in the life cycle of seed prodution and the
88 variation in BCO fungicide activity during the period in wich it accumulates in the cotyledons are not yet studied. To obtain this
89 information, quantitative and activity assays were implemented in this study.

90

91 2. Materials and methods

92 2.1. *Biological material and growth conditions*

93 Dry seeds of white lupin (*Lupinus albus* L.) cvs. Amiga, Energy, Estoril, Ludic, Misak, Multitalia and Rumbo were kindly
94 supplied by CEV/CONVERDE (Cantanhede, Portugal; the commercial manufacturer of BCO for agricultural purposes) and LusoSem
95 (Oeiras, Portugal; a company which commercializes lupin seeds). In addition, four lots of cv. Energy and six different lots of cv. Misak,
96 with different harvest years (1997, 2010, 2013 and 2014) and/or origin sites in Portugal (Alto Alentejo, Baixo Alentejo and Beira Litoral),
97 were kindly supplied by Prof. J. Neves-Martins, responsible for the *Lupinus* germoplasm bank at Instituto Superior de Agronomia,
98 University of Lisbon (ISA). *L. albus* seeds were harvested and stored permanently at -20ºC until used in the assays.

99 Germination was initiated by immersion of the seeds in running tap water for 48 h at 25ºC. The seedlings were then planted in soil
100 and incubated at 25ºC for periods up to 20 days in a 16/8 h light/dark cycle under fluorescent lamps. The plantlets were watered as
101 required with water. In the present study, the seed coats were removed by hand from plantlets aged between 4 and 12-days-old, with intact
102 cotyledons from each cultivar dissected from the axes, weighed, frozen in liquid nitrogen and stored at –80ºC until needed. All samples
103 were analysed in triplicate. Under industrial conditions, these procedures have been automatized.

104 Fungal strains tested for Minimum Inhibitory Concentration (MIC) values were obtained from Instituto Superior de Agronomia's
105 culture collection (*Candida glabrata,* ISA 2163) or isolated by CEV/CONVERDE's lab (*Botrytis cinerea*, CEV 6, isolated from tomato).
106 *B. cinerea* strain was previously identified by sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA
107 (PCR amplification with ITS1 and ITS4 primers).

108 For preparing the inocula, *B. cinerea* was grown on Potato Dextrose Agar (PDA) for 7 days at 25ºC and *C. glabrata* was grown on
109 Glucose Yeast Peptone (GYP) agar for 24 hours at 37ºC.

110 2.2. *Purification of total soluble proteins*

111 Total soluble proteins were extracted and purified by a modification of the Blagrove and Gillespie method (1978), as described by
112 Santos et al. (1997). The cotyledons from *L. albus* plantlets were ground and homogenized with a mortar and pestle, with total
113 cotyledonary protein extracted in globulin solubilizing buffer: 2.5 mL g^{-1} fresh weight, 100 mM Tris-HCl buffer, pH 7.5, containing 10%
114 (w/v) NaCl, 10 mM EDTA and 10 mM EGTA. The homogenized solution was agitated during 30 min at 4ºC, squeezed through two
115 layers of cheesecloth and centrifuged at 15.000 *g* for 1 h at 4ºC. The resulting supernatant was desalted on PD-10 columns (GE
116 HealthCare Life Sciences; disposable desalting Sephadex G-25 Medium columns, 9.1 mL bed volume) previously equilibrated in 50 mM
117 Tris-HCl buffer, pH 7.5.

118 2.3. *Purification of the glyco-oligomer containing blad*

119 The total soluble protein fraction was fractionated and subsequently purified by anion-exchange fast protein liquid
120 chromatography ($ÄKTA_{FPLC}$) on a Resource^{TM} Q column equilibrated with 50 mM Tris-HCl buffer (pH 7.5; GE HealthCare Life
121 Sciences, Uppsala, Sweden; Monteiro *et al*., 2010). Blad (main breakdown product of β-conglutin catabolism) and the other polypeptides

122 which comprise BCO were eluted with a gradient of NaCl (0 to 1 M). For antifungal activity tests, desalting was achieved by dialysis. The
123 presence of blad (20.4 kDa) was detected by SDS-PAGE.

124 2.4. *Electrophoresis*

125 All blad-containing samples were boiled for 3 min in the presence of SDS (2%, w/v) and 2-mercaptoethanol (0.1 M) and subjected
126 to SDS-PAGE in 10% (w/v) acrylamide slab gels as described before (Ferreira et al., 1995b). Total polypeptides in gels were stained with
127 Coomassie Brilliant Blue R.

128 2.5. *Quantification of the glyco-oligomer containing blad*

129 The BCO content of the *Lupinus* extracts was quantified by a spectrophotometrical method, based on BCO molar attenuation
130 coefficient. This value was previously calculated with pure BCO using a modification of the Lowry method (Bensadoun and Weinstein,
131 1976).

132 2.6. *Antifungal activity tests – MIC values*

133 Susceptibility tests of the selected fungal species to BCO were made according to the Reference Methods for Broth Dilution
134 Antifungal Susceptibility Testing guidelines M27-A2 (NCCLS, 2002) and M38-A2 (CLSI, 2008), using the broth microdilution method,
135 with small modifications. As negative controls, experiments were performed in the absence of BCO.
136 ***C. glabrata***: *C. glabrata* was grown as described in Pinheiro et al. (2016, 2018). The suspension was prepared by covering the
137 colonies with 5 mL of sterile 0.9% (w/v) saline (NaCl) solution and diluted to a turbidity equivalent to that of a 0.5 McFarland standard
138 with a spectrophotometer at 640 nm. The suspension was further diluted (1:50) with Potato Dextrose Broth (PDB) medium (pH 7.5),
139 prepared with a double concentration, to yield an inoculum concentration of approximately 1×10^3 cells/mL. One hundred μL was added
140 into the wells of each row containing 100 μL diluted BCO (0.5 mg mL^{-1}). The final volume in each microplate well was therefore 200 μL.
141 Microplates were then incubated at 34ºC without agitation and results were determined after 72 h by visual inspection.

142 ***B. cinerea***: *B. cinerea* was grown as described in Monteiro et al. (2015). The suspension was prepared by covering the fungal
143 colonies with 5 mL of sterile 0.9% (w/v) saline (NaCl) solution containing 0.01% (v/v) polysorbate 20. The suspension was transferred to
144 a sterile tube, mixed in a vortex for 15 s, and the cell density adjusted to 0.4-5.0 $\times 10^6$ CFU/mL by direct counting of spores using a

145 Neubauer chamber. The final inoculum suspension was made by a 1:50 dilution with PDB medium (pH 7.5), prepared with a double
146 concentration, which resulted in a final concentration between 0.4×10^4 to 5.0×10^4 cells/mL. The inoculum size was confirmed by
147 enumeration of CFUs obtained by subculturing on PDA plates. One hundred μL was added into the wells of each row containing 100 μL
148 diluted BCO (0.5 mg mL^{-1}). The final volume in each well was therefore 200 μL. Microplates were then incubated at 25ºC without
149 agitation and results were determined after 72 h by visual inspection.

150 The MIC endpoints were the lowest BCO dilution with no visible growth, as recommended by NCCLS (2002) and CLSI (2008).

151 2.7. *Statistical analysis*

152 A statistical computing tool, R 3.2.0, was used to perform a two-way analysis of variance (ANOVA) on each dataset, with mean
153 separation done by Tukey's range test ($P < 0.05$). When any one of the ANOVA assumptions was not observed, ANOVA results were
154 maintained, but confirmed through non-parametric methods: Kruskal-Wallis test and/or Friedman test.

155

156 **3. Results and discussion**

157 *3.1. Quantification of BCO – The effect of cultivar*

158 The cultivars studied (Amiga, Energy, Estoril, Ludic, Misak, Multitalia and Rumbo) were selected because of their recognized
159 agronomical value and/or high cotyledonary concentration of BCO, as determined by CEV/CONVERDE. The growth and development
160 of lupins during the initial stages of their growing cycle is illustrated in Fig. 1.

161 Quantification of BCO content in *L. albus* plantlets was carried out between days 4 and 12 after the onset of germination. This
162 period is justified by the previously determined abrupt accumulation of this oligomer in the cotyledons of *Lupinus* species during the 4th
163 day after imbibition, maintenance at high levels in these organs during several days, and rapid decline after 12th to 14th days (Ferreira *et*
164 *al*., 1995a; Freitas *et al*., 2007; Monteiro *et al*., 2010, 2015).

165 It remains to be established the molecular mechanism behind this abrupt accumulation of BCO during the fourth day after the
166 onset of germination, although this may be due to structural changes related to proteolysis of β-conglutin, the pre-existing seed storage
167 protein (Ferreira *et al*., 1995b).

168 The results presented in Table 1 show that the selected cultivars exhibit a maximum accumulation of BCO at the 4th day, followed
169 by a progressive and continuous decrease of this oligomer up to the 12th day, where very low BCO values are present ($< 5\ \mu g\ mg^{-1}$ fresh
170 weight). As a result of this decrease, it was observed that the quantitative differences in BCO detected among the different cultivars at day
171 4 vanished along seedling growth due to the proteolytic process.

172 The statistical analysis revealed significant differences between days 4 and 12 after the onset of germination and also among
173 cultivars, as well as the existence of interactions between these two effects as shown by the differential reduction in BCO along time in
174 the different cultivars.

175 The cv. Energy presented the highest BCO accumulation, although cvs. Amiga, Misak and Rumbo were also promising cultivars,
176 for its similar agronomic fitness (verified in field trials – data not shown) and high accumulation of the oligomer ($> 17\ \mu g\ mg^{-1}$ fresh
177 weight).

178 Purified BCO was subjected to gel filtration and SDS-PAGE to confirm its native molecular mass of 210 kDa, as well as its
179 subunit composition and size. The extracts prepared from different varieties contain variable amounts of BCO. To normalize the results
180 and allow for a quantitative comparison of BCO among varieties, the volume of 4-days-old, cv. Energy cotyledon BCO-purified extract
181 containing 20 μg BCO was experimentally calculated to correspond to 22.78 μL. Therefore, 22.78 μL of BCO-purified extract from all
182 cultivars were loaded in the gel, which corresponds to the same mass of 4-days-old cotyledonary fresh weight. As reported earlier (Ramos
183 *et al*., 1997; Monteiro *et al*., 2010), a simple SDS-PAGE analysis (Fig 2) revealed this oligomer to be composed of several polypeptides,
184 the major ones exhibiting molecular masses of 14, 17, 20 (blad), 32, 36, 48 and 50 kDa. A comparison among cultivars showed that BCO
185 polypeptide profile is identical for all of them, although the four cultivars previously indicated (Amiga, Energy, Misak and Rumbo)
186 contain a higher amount of BCO than the other cultivars.

187 As reported before, BCO polypeptide profile suffered changes over time following the 4th day after the onset of germination (Fig 3
188 - A). However, the degradation rate did not seem to be the same for all cultivars studied, but rather seemed to be in accordance with the

189 statistical analysis previously described (Table 1; time:cultivar interaction). This observation was confirmed by SDS-PAGE, where quite
190 different polypeptide profiles were obtained by analysing a fixed quantity of BCO obtained during different days after the onset of
191 germination (Fig 3 – B). The results showed that blad is clearly the most abundant polypeptide band resisting to this kind of proteolytic
192 degradation.

193 *3.2. Quantification of BCO – The effect of edaphoclimatic conditions*

194 BCO was quantified in *L. albus*, but this time comparing factors other than the cultivar, i.e. for the same cultivar (Energy or
195 Misak) seeds produced in different years (and therefore subjected to storage at -20ºC for different lengths of time) and in different regions
196 were studied. This assay showed a great heterogeneity among and within the seed lots, i.e., heterogeneity among different batches/lots of
197 the same cultivar and heterogeneity among plants belonging to the same batch (origin site and harvest year – Table 2). Although standard
198 error values were always quite high, these results seem quite consistent, given the high significance of seed lot and cultivar factors, as
199 well as the existence of interactions between these factors.

200 A careful analysis of the data presented in Table 2 suggests that BCO levels in 4-day-old *L. albus* cotyledons are dependent on the
201 cultivar and on both the year and the region (collectively on the edaphoclimatic conditions) where the seeds were produced, and do not
202 seem to depend on the length of storage time frozen at -20ºC. Therefore, the results may be due to effects from cultivar, fertilization, site
203 and region of seed production, harvest year, productivity and/or storage conditions, factors that influence in general the composition and
204 quantity of cellular components (Bosworth *et al*., 2013; Halvorson *et al*., 2003; Kallio *et al*., 2002; Sluis *et al*., 2001), and which certainly
205 conditioned the plants that produced the seeds analysed in the present work. These new and unexpected results greatly influence the level
206 of extractable BCO, being of enormous interest to the BCO production industry.

207

208 *3.3 BCO antifungal activity tests – MIC value determinations*

209 Besides functioning as a cotyledonary storage globulin in *L. albus*, BCO fulfils other physiological roles in the plant, based on the
210 highly remarkable number of distinct biochemical properties exhibited by this oligomer, most notably its catalytic activities, lectin activity
211 and resistance to denaturation (Monteiro *et al*., 2015).

212 To determine BCO anti-fungal activity, one experiment was conducted on *Botrytis cinerea*, a necrotrophic fungus that affects
213 many plant species, and on *C. glabrata*, an haploid yeast of the genus *Candida* which is a closely related opportunistic human pathogen
214 and a model organism often used in antifungal activity experiments (NCCLS, 2002; CLSI, 2008). BCO was obtained from the cotyledons
215 of 3-, 4-, 5-, 8-, 12- and 16-days-old *L. albus* plantlets, from cvs. Energy and Rumbo, respectively first and second cultivars with the
216 highest accumulation of BCO in the previous quantitative assay (Table 1).

217 The susceptibility of pathogenic fungi to the oligomer was assessed *in vitro* by the determination of the MIC, i.e., the lowest BCO
218 concentration that inhibits visible growth of a fungal strain. BCO antifungal activity was tested against the oligomer and the results are
219 shown in Table 3.

220 The MIC value range was relatively large, but essentially this experiment evidenced quite new and practical results, i.e., BCO
221 demonstrated anti-fungal activity between 3-5 days after the onset of germination (no statistically significant differences) and,
222 surprisingly, no antifungal activity in the cotyledons from 8-, 12- and 16-days-old plantlets (Table 3). The range of BCO concentrations
223 tested were specified in the international standards for this test (CLSI, 2008), and MIC values were also within the common values for
224 this type of test (Bueno *et al*., 2010; Silva Barros *et al*., 2007). Although the doses (when expressed in units of mass) of BCO required for
225 fungal inhibition *in vitro* were higher than those usually required for other antifungal drugs, it should be noted that BCO molecular mass
226 was also substantially higher (210 kDa; typically by almost two orders of magnitude) that that of commercial fungicides, which means
227 that when expressed on a molar basis or on the number of molecules required to exhibit inhibitory effect, BCO antifungal activity was
228 quite similar to that of the available drugs (Monteiro *et al*., 2015).

229

230 **4. Conclusions**

231 Several parameters were addressed in the present study. The cultivar, and the year and place of seed production (collectively
232 defining the edaphoclimatic conditions) on BCO amount and the age of the seedling cotyledons on BCO fungicidal activity. The
233 innovative results obtained were both unexpected and surprising, but of great relevance in what concerns BCO industrial production.

234 Two major conclusions may be drawn from the results presented in this work:

235 - The amount of extractable BCO is not directly related to its fungicidal potency, as they both vary widely in cotyledons during
236 seedling growth in an apparently independent way. Thus, for example, BCO is still present in cotyledons between days 8 and 12 after the
237 onset of germination but is seems to be devoid of fungicide activity. Consequently, judging BCO fungicide potency by calculating BCO
238 amount is not an appropriate choice.

239 - The amount of extractable BCO seems to depend to a much greater extent on the edaphoclimatic conditions prevailing during
240 vegetative growth and seed formation than on the Lupinus cultivar.

241 Specific and detailed physiological and agronomical studies should therefore be conducted to identify the edaphoclimatic
242 conditions during plant growth, as well as the seed storage conditions after harvest and the germinating and plantlet growth duration and
243 conditions, in order to optimize all these variables in a way to maximize extractable BCO fungicidal activity.

244 As BCO industrial production requires massive germination and plantlet growth of sweet Lupinus plants for ca. 8 days, followed
245 by a suitable extraction methodology, incrementing/maximising the amount of extractable BCO is therefore highly relevant and comes as
246 a priority for the BCO industry.

247

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Table 1

Content of BCO (µg mg^{-1} fresh weight) in the plantlet cotyledons of seven *L. albus* cultivars.

Time (DAG)	BCO (µg mg^{-1} fresh weight)													
	AMIGA		ENERGY		ESTORIL		LUDIC		MISAK		MULTITALIA		RUMBO	
	Mean (SE)	HG	Mean (SE)	HG	Mean (SE)	HG	Mean (SE)	HG	Mean (SE)	HG	Mean (SE)	HG	Mean (SE)	HG
4	17.32 (0.07)	a	31.67 (0.05)	a	13.13 (0.08)	a	15.21 (1.09)	a	22.12 (0.55)	a	9.35 (0.33)	a	25.67 (0.26)	a
5	17.14 (0.05)	a	26.49 (0.05)	b	13.20 (0.16)	a	13.48 (0.24)	ab	16.16 (0.57)	b	7.20 (0.67)	ab	21.67 (0.12)	b
6	16.41 (0.49)	a	14.86 (0.82)	c	9.93 (0.69)	b	11.96 (0.64)	b	15.04 (0.03)	b	7.95 (0.21)	ab	17.89 (0.63)	c
7	13.46 (0.82)	b	13.73 (0.30)	c	7.70 (0.13)	c	5.78 (0.13)	c	10.32 (0.29)	c	6.64 (0.07)	bc	11.70 (0.29)	d
8	11.57 (0.03)	bc	10.96 (0.46)	d	3.29 (0.09)	d	4.53 (0.33)	cd	8.45 (0.11)	d	6.22 (0.20)	bc	12.30 (0.04)	d
9	9.96 (0.88)	c	9.13 (0.40)	d	3.11 (0.06)	d	4.75 (0.35)	cd	9.27 (0.08)	d	5.80 (0.18)	bc	9.09 (0.09)	e
10	7.88 (0.18)	d	9.13 (1.19)	d	4.28 (0.05)	d	2.93 (0.26)	de	6.62 (0.53)	e	5.75 (0.18)	bc	7.73 (0.03)	f
11	4.55 (0.19)	e	5.66 (0.55)	e	2.21 (0.02)	e	3.02 (0.19)	de	4.26 (0.18)	f	4.50 (0.30)	cd	8.32 (0.13)	f
12	4.98 (0.05)	e	4.56 (0.14)	e	2.18 (0.04)	e	1.41 (0.03)	e	4.85 (0.08)	f	2.49 (1.05)	d	4.27 (0.10)	g

ANOVA	Time factor	Time:Cultivar interaction	Cultivar factor
F	1138.99	79.77	504.92
P-value	< 2E-16	< 2E-16	< 2E-16

DAG - Days after the onset of germination   HG - Homogeneous Groups   Different letters indicate significant differences among DAG ($P < 0.05$)

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Table 2
BCO quantitative assay from various seed lots of *L. albus* cvs. Energy and Misak in 4-day-old cotyledons produced under different edaphoclimatic conditions (different harvest years and/or origin sites in Portugal).

Lot	BCO (µg mg^{-1} fresh weight)			
	ENERGY		MISAK	
	Mean (SE)	Homogeneous Groups	Mean (SE)	Homogeneous Groups
Alto Alentejo	8.97 (5.18)	b	51.26 (29.60)	a
Baixo Alentejo	7.49 (4.32)	c	36.64 (21.16)	b
Beira Litoral	10.16 (5.86)	a	36.72 (21.20)	b
ISA 2014	6.83 (3.94)	d	5.89 (3.40)	e
ISA 2010	-	-	12.92 (7.46)	d
ISA 1997	-	-	33.80 (19.52)	c
ANOVA	Lot Factor	Time:Cultivar interaction	Cultivar factor	
F	865.8	760.2	4961.2	
P-value	< 2E-16	< 2E-16	< 2E-16	

335 Different letters indicate significant differences among lots ($P < 0.05$)

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Table 3

BCO antifungal activity. *L. albus* cvs Energy and Rumbo seeds days after the onset of germination. No antifungal activity was detected for the negative control (absence of BCO).

	Minimum Inhibitory Concentration (MIC, µg mL^{-1})							
Fungal Species	*Botrytis cinerea*				*Candida glabrata*			
Time (DAG)	ENERGY		RUMBO		ENERGY		RUMBO	
	Mean (SE)	HG	Mean (SE)	HG	Mean (SE)	HG	Mean (SE)	HG
3	93.75 (31.25)	b	270.83 (126.72)	b	208.33 (41.67)	b	125.00 (62.50)	b
4	135.42 (63.36)	b	135.42 (63.36)	b	93.75 (31.25)	b	208.33 (41.67)	b
5	125 (0)	b	541.67 (253.45)	b	145.83 (55.12)	b	333.33 (83.33)	b
8	> 500	a	> 500	a	> 500	a	> 500	a
12	> 500	a	> 500	a	> 500	a	> 500	a
16	> 500	a	> 500	a	> 500	a	> 500	a
ANOVA	Time factor		Cultivar factor		Time factor		Cultivar factor	
F	33.75		5.81		33.65		5.8	
P-value	3.07E-11		0.02		3.19E-11		0.02	

338 DAG - Days after the onset of germination HG - Homogeneous Groups Different letters indicate significant differences among DAG ($P < 0.05$)

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340 Fig. 1

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345 Fig. 2

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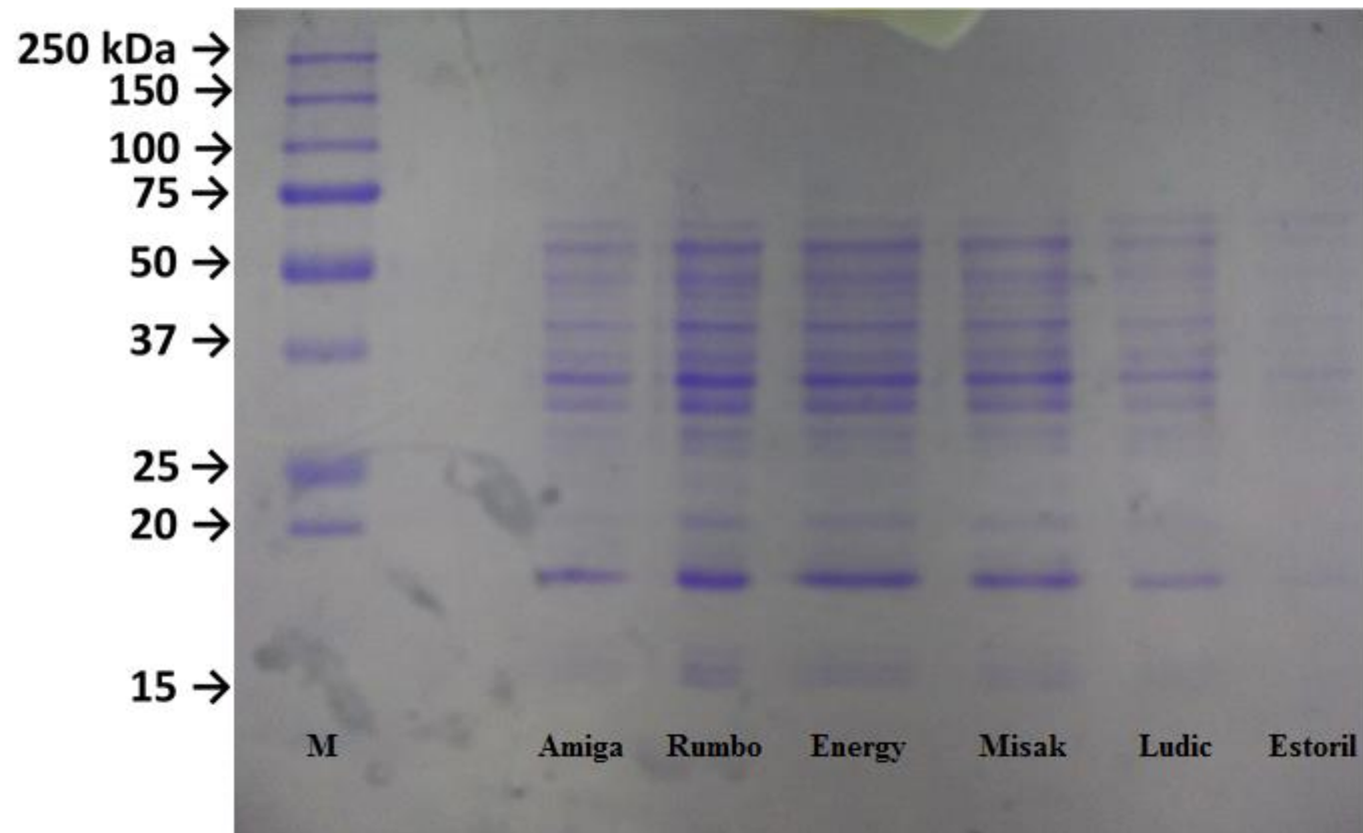

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351 Fig. 3A

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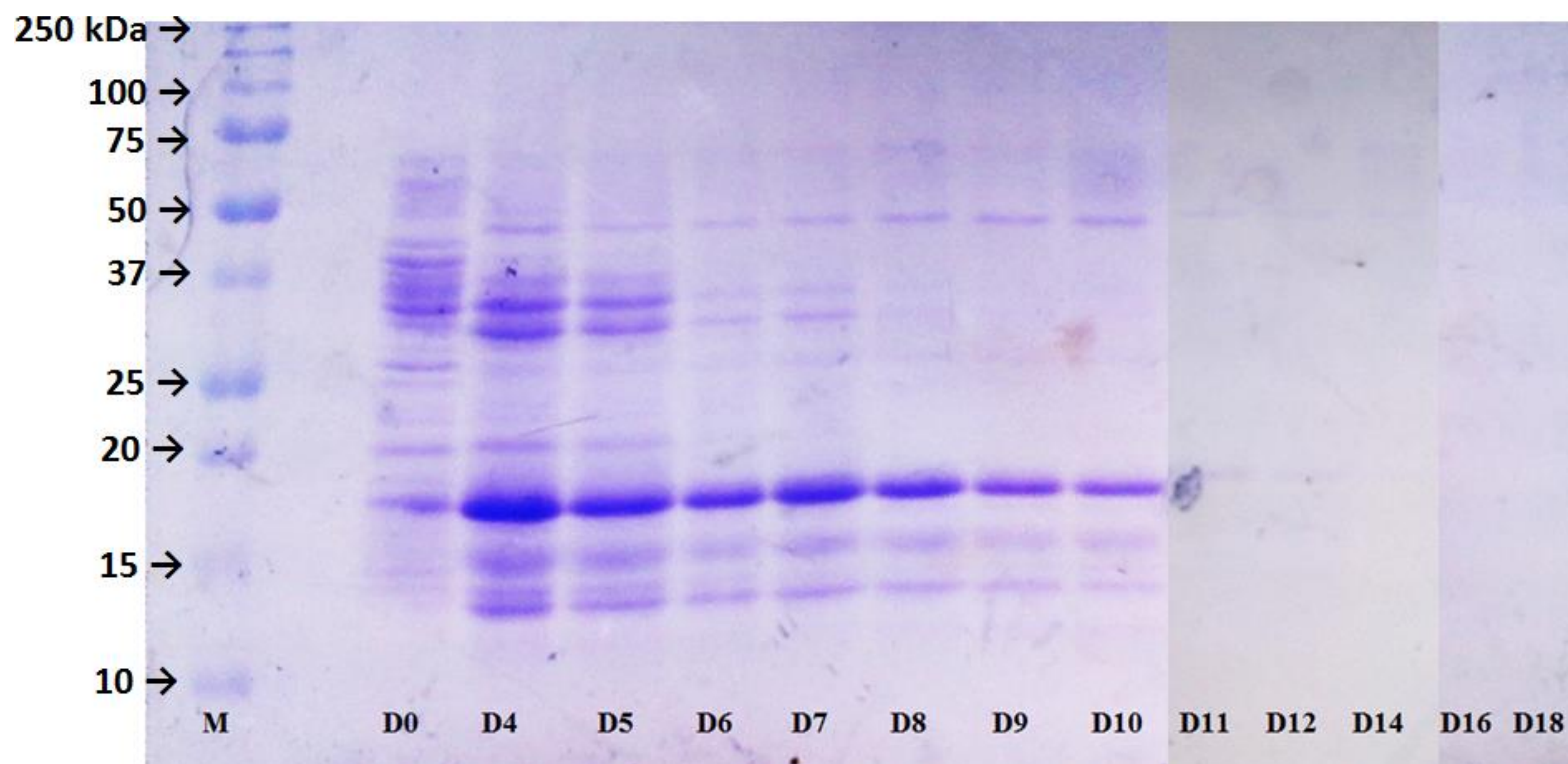

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357 Fig. 3B

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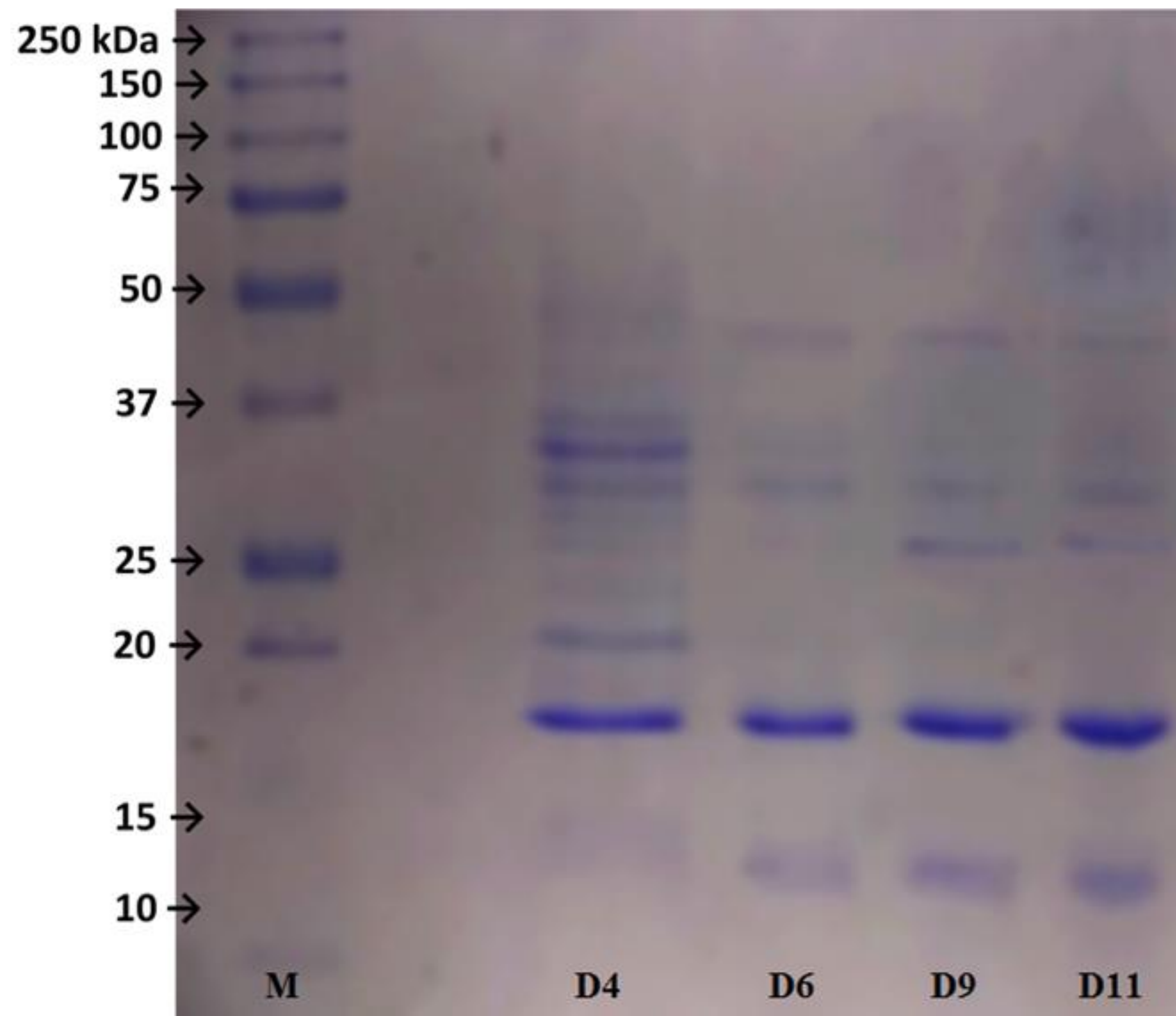

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363 **Fig. 1.** Development stages of *L. albus* during the first 20 days after the onset of germination The green line indicates the presence of
364 BCO in the cotyledons.

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366 **Fig. 2.** Comparative quantitative and structural SDS-PAGE analyses of BCO purified from the cotyledons of six *L. albus* cultivars 4 days
367 after the onset of germination. *L. albus* seeds were germinated and grown for four days and BCO extracted, purified, analyzed by one-
368 dimensional SDS-PAGE and stained for total polypeptides. The volume loaded in each lane (22.78 µL) corresponded to 20 µg of BCO for
369 the cultivar Energy. The cultivars analysed are indicated in the bottoom of the gel. Molecular masses of standards are indicated in kDa.

370

371 **Fig. 3.** One-dimensional structural analysis of BCO from *L. albus*, cv. Energy. *L. albus* seeds were germinated and grown for up to 18 days
372 and BCO extracted, purified, analyzed by one-dimensional SDS-PAGE and stained for total polypeptides. **(A)** – Purified BCO extract (22.78
373 µL; see legend to Figure 2) was loaded in each lane. **(B)** – Purified BCO (20 µg) was loaded in each lane. Days after the onset of germination
374 are indicated in the bottoom of the gel. Molecular masses of standards are indicated in kDa.

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