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1 # Androgenesis-inducing stress treatments change phytohormone levels
2 # in anthers of three legume species (Fabaceae)

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28 **Abstract**

29 Legumes are recalcitrant to androgenesis and only recently protocols were developed for pea (*Pisum sativum* L.) and
30 chickpea (*Cicer arietinum* L.) albeit with low regeneration frequencies. Androgenesis is thought to be mediated
31 through abscisic acid (ABA) but other phytohormones such as auxins, cytokinins and gibberellins have also been
32 implicated. In view of improving protocols, hormone content of anthers was measured after exposure to cold,
33 centrifugation, electroporation, sonication, osmotic shock or various combinations thereof using an analytical mass
34 spectrometery-based analysis. Auxin had a key function during the induction process. High concentrations of IAA-
35 Asp, a putative IAA metabolite, accumulated during the application of the different stresses in pea. In chickpea, the
36 IAA-Asp concentration increased 30 fold compared to those of pea but only during the osmotic shock treatment and
37 likely as a result of the presence of exogenous IAA in the medium. In contrast, no treatment showed such an
38 increase in auxin content in the recalcitrant lentil (*Lens culinaris*). Of the various cytokinins monitored, only cZR
39 increased after centrifugation and electroporation in pea and possibly chickpea. No bioactive gibberellins were
40 detected indicating that this hormone group is probably not linked to androgenesis. In contrast to the other stresses,
41 osmotic shock treatment caused a sharp reduction in the levels of all hormones analyzed, with the exception of
42 auxins in chickpea. A short period of low hormone content might be a necessary transition phase required for
43 androgenesis induction of legumes.

44 **KEYWORDS**: Abscisic acid, auxin, cytokinin, gibberellin, centrifugation, electroporation, sonication, osmotic
45 shock

46

47 **Abbreviations**: **ABA**, Abscisic acid; **ABA-GE**, ABA-glucose ester; **7'OH-ABA**, 7'-Hydroxy-ABA; **BAP,** Benzyl
48 amino purine; C, Centrifugation; **4-Cl-IAA,** 4-chloroindole-3-acetic acid; 2,4-**D**, 2,4-dichlorophenoxyacetic acid;
49 **DH,** Doubled-haploid; **DPA,** Dihydrophaseic acid; **E,** Electroporation; **GA**$_{1\text{-}53}$, Gibberellins 1 – 53; **IAA,** Indole-3-
50 acetic acid; **IAA-Ala,** N-(Indole-3-yl-acetyl)-alanine; **IAA-Asp,** N-(Indole-3-yl-acetyl)-aspartic acid; **IAA-Glu**, N-
51 (Indole-3-yl-acetyl)-glutamic acid; **IAA-Leu**, N-(Indole-3-yl-acetyl)-leucine; **IBA**, Indole-3-butyric acid; **2iP**,
52 Isopentenyladenine; **iPA**, Isopentenyladenosine; **NAA**, 1-naphthaleneacetic acid; **O,** Osmotic shock; **PA**, Phaseic
53 acid; S, Sonication; **Z**, Zeatin; **ZOG**, Zeatin-O-glucoside; **dhZ**, Dihydro-zeatin; **ZR**, Zeatin riboside

Introduction

Androgenesis is the development of plants containing only paternally derived chromosomes. Protocols for the development of double-haploid plants through androgenesis have been published for more than 200 species (Wedzony et al., 2009), but legumes are considered recalcitrant although recent breakthroughs have been made in the development of protocols for pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.) (Croser et al., 2006, Grewal et al., 2009, Ochatt et al., 2009). A soybean (*Glycine max* L. Merr.) androgenesis protocol has stalled at the embryo induction stage (Kaltchuk-Santos et al., 1997) and there is no protocol available for lentil (*Lens culinaris* Medik.).

In many species the induction of androgenesis requires the application of abiotic stresses such as heat, cold, or osmotic shock (Shariatpanahi et al., 2006). Cold stress is known to alter the division of the microspore nuclei from asymmetrical to symmetrical and this switch has been considered as one of the main routes in the formation of haploid cells and embryos (Rashid and Street, 1974; Smykal, 2000; Zaki and Dickinson, 1990). Cold treatment of plants or floral organs is thought to sustain microspore viability and to provide a period of carbohydrate starvation concomitant with an increase in the abscisic acid (ABA) levels (Żur et al., 2008). A 2 to 30 day cold stress of flower buds was important for induction of androgenesis in some French pea varieties, but more than 3 or 4 days of cold stress was detrimental in chickpea (Grewal et al., 2009, Ochatt et al., 2009). Osmotic stress is mediated through carbohydrates such as sucrose or maltose; they function as energy sources as well as osmotic regulators and influence both androgenic induction and embryo development (Ferrie et al., 1995; Hoekstra et al., 1993; Lionneton et al., 2001). Dunwell and Thurling (1985) observed a beneficial effect of a high level of sucrose on canola (*Brassica napus* L.) microspores and attributed it to the fact that a medium containing 17% sucrose generated an osmotic potential similar to that of an anther homogenate. A lower level of sucrose was subsequently required for a sustained level of microspore division (Ferrie et al., 1999; Lionneton et al. 2001). Similarly, carbohydrate requirements for pea and chickpea required a switch from 17% to a lower level of sucrose (Grewal et al., 2009; Ochatt et al., 2009).

The application of physical stressors such as centrifugation, electroporation, or sonication has been shown to improve the androgenic potential of various species. Centrifugation increased androgenesis induction of cultured anthers in *Datura innoxia* and in tobacco (*Nicotiana tabacum* L.) (Sangwan-Norreel, 1977; Tanaka, 1973).

81 Similarly, centrifugation showed a positive effect on androgenesis induction in chickpea and pea (Grewal et al., 82 2009; Ochatt et al., 2009). Electroporation appears to exert its effect through the introduction of medium 83 components into the cells although a direct effect of electric pulses on androgenesis cannot be excluded since 84 electric pulses were shown to stimulate protoplast growth and regeneration ability (Rech et al., 1987; Ochatt et al., 85 1988). Electroporated microspores developed embryos faster for *Gingko biloba* L. (Laurain et al., 1993), canola 86 (Jardinaud et al., 1993), pea (Ochatt et al., 2009), and chickpea (Grewal et al., 2009). Similar to electroporation, 87 sonication causes microcavity formation and collapse resulting in reversible permeability changes of cell membranes 88 and thus facilitating the uptake of medium components (Fizzell, 1988; Joersbo and Brunstedt, 1992). Sonication 89 using 40 KHz for 30 s enhanced androgenetic callusing in pea but only if combined with other stresses (Ribalta et 90 al., 2010). The key for overcoming androgenetic recalcitrance in pea and chickpea was the pyramiding of multiple 91 stressors including cold, osmotic shock, centrifugation, electroporation, and sonication (Grewal et al., 2009; Ochatt 92 et al., 2009, Ribalta et al., 2010). The requirement of three or more stresses is unusual for androgenesis indicating 93 that the mechanism in legumes is poorly understood.

94 Triggering embryo development in non-ovum cells is linked to stress, which in turn is linked to changes in 95 endogenous hormone content. Abscisic acid is the 'stress' hormone since it plays a major role in adaptation of 96 plants to adverse conditions (Kikuchi et al., 2006; Segui-Simarro and Nuez, 2008; Zavattieri et al., 2010; Żur et al., 97 2008). However, various other plant hormones are also affected by stress including auxins (IAA), cytokinins 98 (zeatin), gibberellins (GA), or reactive oxygen species (Maraschin et al., 2005, 2006; Zavattieri et al., 2010). 99 Cytokinin concentrations generally decrease in response to stress (Brugiere et al., 2003), whereas auxin responses 100 vary (Jain and Khurana, 2009). Although gibberellins are not linked to abiotic stress, gibberellins prevent the stress 101 response signal pathway being triggered (Gao et al., 2010). Interestingly, this pathway is down-regulated by heat 102 and cold stress, but up-regulated by salt and drought stress indicating gibberellins may be effected by some stresses.

103 Given the need for effective androgenesis protocols in legumes and the evidence supporting the 104 phytohormonal regulation of such a process, we have measured the concentrations of ABA, auxins, cytokinins and 105 gibberellins in anthers of pea, chickpea, and the recalcitrant lentil after various stress treatments. We reasoned that 106 ABA and likely auxin concentrations would increase as a result of the different stresses. Furthermore, we expected 107 that pyramiding the different stresses would have an additive effect since only the combination of the different

108 stresses led to induction and regeneration. As a result of this investigation, we have established a baseline for the
109 role of the four hormones classes during androgenesis induction of legumes but also show that osmotic shock
110 treatment seems to have a different function compared to the other stresses.

111 **Materials & methods**

112 *Donor plant growth*

113 Plants were grown in the University of Saskatchewan greenhouses located in Saskatoon, Canada (52° 10.2'
114 N, 106° 43.2 W). The regime consisted of 18 h light and 6 h dark with supplementation from high pressure sodium
115 lamps. Temperatures were set to 22°C day/20°C night. Donor plants were grown in Sunshine® mix No. 4 with 5
116 plants/11 L pot for chickpea and pea and Sunshine® mix No. 3 for lentil with 3 plants/ 8 L pot.

117 *Flower buds and anthers*

118 Flower clusters of chickpea containing different sizes of buds were picked from cultivar CDC Xena, from
119 which buds ranging from 2.0 to 3.0 mm in length were selected for experiments (Grewal et al. 2009). Buds ranging
120 in size from 6.0 to 8.0 mm in length were collected from pea (*Pisum sativum* L.) cultivars CDC April and Frisson
121 (Ochatt et al. 2009). Flower buds from lentil (*Lens culinaris* Medic.) cultivar CDC Maxim were harvested when
122 they were 1.5 to 2.0 mm in length. For cold treatments at 4°C, buds or clusters were kept in Petri® dishes on
123 moistened filter paper. Microspores from all species were at the uni-nucleate stage (confirmed by DAPI staining).
124 Approximately 500 mg of fresh weight was collected for each sample. All material was kept on ice during anther
125 dissection and then freeze-dried. Buds required for the osmotic shock treatment were sterilized with 2% buffered
126 bleach solution for 20 min, rinsed three times with sterile water, and anthers removed under sterile conditions
127 (Grewal et al., 2009).

128 *Stress treatments*

129 Stress treatments for chickpea were applied according to Grewal et al. (2009) and according to Ochatt et al.
130 (2009) for field pea except that the cold treatment of buds lasted 7 days (cold control) and the osmotic shock
131 treatment was reduced from 7 to 3 days. For the control treatments, anthers were dissected out and immediately
132 frozen at -80°C and later dried. For all other stress treatments, flower buds were kept at 4°C for 3 or 7 days and then

133 anthers were dissected out. Chickpea anthers were first transferred to a 0.4 cm electroporation cuvette filled with 1
134 ml RM-IK17 (containing 22.83 µM IAA and 1.86 µM kinetin) and then either centrifuged (C) in an ICE Centra CL-
135 2 centrifuge for 10 min at 168 *g*, or electroporated (E) with 1250 V/cm, 25 Ω and 25 µF using three pulses, or kept
136 in the same RM-IK17 medium for four days (O), or a combination thereof (C+O, E+O, C+E+O) (see Grewal et al.,
137 2009 for details).

138 Regarding pea, anthers were transferred to a 0.2 cm cuvette with 1 ml HSO-17% sucrose, followed by
139 centrifugation at 4°C (168 *g* for 10 min), or electroporation using 2500 V/cm, 50 Ω, and 100 µF or sonication (S)
140 with 42 KHz for 30 s (Branson Ultrasonic cleaners® Model 2510), or kept in osmotic shock medium (30 anthers in
141 6 ml HSO-17% sucrose), or a combination thereof as described in Ochatt et al. (2009) and Ribalta et al. (2010).

142 Preliminary experiments showed that stress treatments similar to those of pea and chickpea were lethal for
143 lentil microspore viability. Hence, only treatments that had confirmed microspore survival in preliminary
144 experiments were selected for hormone profiling. After dissection, lentil anthers were either centrifuged at 168 *g* for
145 3 min, or electroporated with 625 V/cm, 25 Ω, and 25 µF, or treated with osmotic shock (500 anthers per plate with
146 4.5 ml modified B5 medium plus 9% sucrose), or kept for 3 or 7 days in cold, or a combination thereof.

147 *Hormone analysis*

148 Quantification of ABA, cytokinins, auxins and GAs in control and stressed chickpea, field pea and lentil
149 anthers was conducted at the Plant Biotechnology Institute of the National Research Council of Canada by UPLC-
150 ESI-MS/MS (http://www.nrc-cnrc.gc.ca/eng/facilities/pbi/plant-hormone.html). The following hormones were
151 quantified (1) ABA and ABA metabolites (*cis* and *trans* abscisic acid [ABA], phaseic acid [PA], dihydrophaseic
152 acid [DPA], 7'-hydroxy ABA, *neo*-phaseic acid [*neo*PA], abscisic acid glucose ester [ABA-GE]; (2) auxins (indole-
153 3-acetic acid [IAA], indole-3-butyric acid [IBA], indole-3-acetic acid aspartate [IAA-Asp], indole-3-acetic acid
154 glutamate [IAA-Glu], N-(Indole-3-yl-acetyl)-alanine [IAA-Ala], N-(Indole-3-yl-acetyl)-leucine [IAA-Leu]); (3)
155 cytokinins (*cis* and *trans* zeatin [Z], *cis* and *trans* zeatin riboside [ZR], *cis* and *trans* zeatin-O-glucoside [ZOG],
156 dihydrozeatin [dhZ], dihydrozeatin riboside [dhZR], isopentenyl adenine [2iP], isopentenyl adenosine [iPA] and (4)
157 gibberellins (GA1, 3, 4, 7, 8, 9, 19, 20, 24, 29, 34, 44, 51 and 53). Deuterated forms of the hormones were used as
158 internal standards. They were either synthesized according to Abrams et al. (2003) and Zaharia et al. (2005) (d_3-

159 DPA, d_5-ABA-GE, d_3-PA, d_4-7'-OH-ABA, d_3-*neo*PA, d_4-ABA, d_4-*trans*-ABA, d_3-IAA-Leu, d_3-IAA-Ala, d_3-IAA-
160 Asp, d_3-IAA-Glu) or purchased from Cambridge Isotope Laboratories (Andover, MA, d_5-IAA), Olchemim Ltd
161 (Czech Republic, d_3-dhZ, d_3-dhZR, d_5-Z-O-Glu, d_6-iPA and d_6-2iP) and Research School of Chemistry, Australian
162 National University (Canberra, Australia, d_2-GAs 1, 3, 4, 7, 8, 9, 19, 20, 24, 29, 34, 44, 51 and 53). The extraction
163 and purification of samples was performed following an existing method described in Chiwocha et al. (2003, 2005)
164 to which improvements were made (Han et al, unpublished).

165

166 Analysis was performed on a UPLC/ESI-MS/MS utilizing a Waters ACQUITY UPLC system, equipped
167 with a binary solvent delivery manager and a sample manager coupled to a Waters Micromass Quattro Premier XE
168 quadruple tandem mass spectrometer via a Z-spray interface. MassLynx™ and QuanLynx™ (Micromass,
169 Manchester, UK) were used for data acquisition and data analysis. The procedure for quantification of multiple
170 hormones has been described in detail by Chiwocha et al. (2003, 2005). Samples were injected onto an ACQUITY
171 UPLC® HSS C18 SB column (2.1x100 mm, 1.8 µm) with an in-line filter and separated by a gradient elution of
172 water containing 0.02% formic acid against an increasing percentage of a mixture of acetonitrile and methanol
173 (volume ratio: 50:50). Briefly, the analysis utilizes the Multiple Reaction Monitoring (MRM) function of the
174 MassLynx v4.1 (Waters Inc) control software. The resulting chromatographic traces are quantified off-line by the
175 QuanLynx v4.1 software (Waters Inc) wherein each trace is integrated and the resulting ratio of signals (non-
176 deuterated/internal standard) is compared with a previously constructed calibration curve to yield the amount of
177 analyte present . Calibration curves were generated from the MRM signals obtained from standard solutions based
178 on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as
179 described by Ross et al. (2004). The QC samples, internal standard blanks and solvent blanks were also prepared and
180 analyzed along each batch of tissue samples.

181 *Statistical analysis*

182 Experiments were replicated three times unless otherwise indicated; and means ± standard errors (SE) were
183 calculated. Data reported in nM g^{-1} dry weight (DW).

184 **Results**

185 **Field pea (*Pisum sativum* L.) var. CDC April and Frisson**

186 In CDC April and Frisson, the ABA concentration was highest in control anthers with 2.1 and 2.6 nM g^{-1}
187 DW, respectively. All stress treatments reduced ABA content (Table 1). Centrifugation increased t-ABA levels in
188 CDC April whereas in Frisson, this isomer increased after cold and combinations of cold with electroporation,
189 centrifugation, or sonication. The presence of high levels of DPA and PA in both species indicates that a rapid
190 breakdown of ABA is taking place via the 8'-hydroxylation pathway. For CDC April, DPA spiked at 131.0 nM g^{-1}
191 DW when no stress was applied (control) whereas PA peaked after cold stress with 173.2 nM g^{-1} DW. In Frisson,
192 the highest PA level was measured in control anthers with 189.1 nM g^{-1} DW and the highest DPA level of 51.2 nM
193 g^{-1} DW in the cold plus centrifugation treatment. *Neo*-PA levels were highest in the control treatment of both
194 varieties but levels indicate that they played only a minor role. The values for 7'OH-ABA and ABA-GE were near
195 detection limits and thus were of little significance to the androgenesis process. However, the treatments involving
196 osmotic shock differed from the other treatments since ABA and its catabolites attained very low values especially
197 in the combination of cold +C+E+S+O (Table 1).

198 Regarding auxin, IBA was not detected in any of the three species. The IAA concentration of CDC April
199 was 1.6 nM g^{-1} DW for both control and cold-treated anthers (Table 2). This concentration increased about two fold
200 after the cold plus centrifugation treatment. The other stress treatments only slightly raised IAA levels compared to
201 the control. IAA-Asp content peaked after application of all stresses except osmotic shock with the highest value of
202 42.7 nM g^{-1} DW attained in the cold+C+E treatment. In contrast, combining the different stresses with osmotic
203 shock treatment (cold+E+C+S+O) led to very low levels of IAA and its catabolites (Table 2). IAA-Glu, another
204 auxin catabolite, was only detected in small amounts whereas the reversible IAA storage forms, IAA-Ala and IAA-
205 Leu, were either not present or only detected in minute amounts close to detection levels. The IAA concentration of
206 var. Frisson was generally lower than in CDC April (Table 2). Compared to control anthers, centrifugation only
207 doubled the IAA concentration in this cultivar; it did not quadruple it as in CDC April indicating that the two
208 genotypes respond somewhat differently to this stress. In contrast, cold plus sonication was a more effective stress
209 treatment in Frisson which increased the IAA concentration to 2.8 nM g^{-1} DW although this concentration was not
210 significantly different from the cold +C or cold +E treatments. IAA-Leu and IAA-Ala were not detected in these

211 samples. Frisson also displayed very high concentrations of IAA-Asp of up to 45.7 nM g^{-1} DW as a result of the
212 various stress treatments except those involving osmotic shock (Table 2). Similar to CDC April, the latter treatment
213 again reduced the content of auxin and its catabolites to low levels.

214 The free base bioactive cytokinins tZ, cZ, or dhZ, were either not detected or present in very low
215 concentrations in anthers of both varieties. The major forms were cZR, iPA as well as 2-iP (Table 3). The cis
216 isomer of ZR spiked in the centrifugation treatment of CDC April with 1.8 nM g^{-1} DW. Compared to the control,
217 cZR was significantly higher after application of stress in Frisson but the difference among the stress treatments was
218 minor. In CDC April, the 2iP concentrations were lower in the stress treatments (0.5 - 0.7 nM g^{-1} DW) compared to
219 the control (0.9 nM g^{-1} DW) but similar or slightly higher for Frisson. The osmotic shock treatment again either
220 completely removed or reduced the different cytokinin forms to very low levels in both varieties (Table 3).

221 No bioactive gibberellins (GA_1, GA_3, GA_4 and GA_7) were detected in pea anthers of both varieties, only
222 precursors (GA_9, GA_{19}, GA_{20}, GA_{24}, GA_{34}, GA_{44} and GA_{53}) and catabolites (GA_{29}, and GA_{51}). A notable genotype
223 difference between the two field pea cultivars tested was that CDC April had GA precursors for both the non-13-
224 hydroxylation pathway (GA_9 and GA_{24} are precursors for GA_4) and the early-13-hydroxylation pathway (GA_1),
225 whereas Frisson only contained precursors related to the latter pathway. Control anthers contained the widest variety
226 of gibberellins (Table 4). After the various stress treatments, GA_{19} was the major component in both varieties with
227 concentrations of 2.0 nM g^{-1} DW in the cold +C+E treatment of CDC April and 1.9 nM g^{-1} DW in the cold treatment
228 of Frisson. Similar to the other hormones, the osmotic shock treatment reduced the concentration of this hormone
229 group to zero or to minute amounts of GA_{19} (Table 4).

230

231 **Chickpea (*Cicer arietinum* L.) var. CDC Xena**

232 The ABA content of control anthers (10.4 nM g^{-1} DW) was significantly higher than after the different
233 stress treatments. Application of a 3-day cold treatment to flower buds reduced this concentration by about 50%
234 (Table 1). Both centrifugation and electroporation further reduced ABA content. Compared to field pea, DPA levels
235 of cold-treated anthers were much lower in chickpea and reached only a maximum of 6.3 nM g^{-1} DW. PA
236 concentrations in control and cold-treated anthers were also lower with maximum values of 26.1 and 21.3 nM g^{-1}

237 DW, respectively. However, the combination of cold with either centrifugation or electroporation resulted in very
238 high PA levels of more than 100 nM and moderate DPA concentrations (Table 1). The various combinations with
239 osmotic shock reduced ABA and its metabolites to very low levels (Table 1).

240 Regarding auxins, control anthers contained on average only 0.6 nM g^{-1} DW IAA as well as 0.07 nM g^{-1}
241 DW of IAA–Asp. The IAA storage forms, IAA-Ala and IAA-Leu, as well as the conjugate IAA-Glu were not
242 detected (Table 2). Three days of cold treatment of buds slightly reduced the auxin content. In contrast to field pea,
243 the medium used during centrifugation, electroporation and osmotic shock treatments contained 22.83 μM IAA.
244 Since the effect of the centrifugation treatment is thought to be mediated through an increase in the auxin content,
245 this was tested by analyzing anthers which had been centrifuged either with or without IAA in the medium. Data
246 shows that the cold plus centrifugation treatment without IAA (0.43 nM g^{-1} DW) was not different from the cold
247 control indicating that centrifugation itself does not increase auxin content of chickpea anthers. Adding IAA to the
248 medium during centrifugation increased the concentration about 13 times to 5.57 nM g^{-1} DW. Similarly, adding IAA
249 during the electroporation treatment also led to an increased IAA concentration (3.37 nM g^{-1} DW) compared to
250 electroporation without IAA (0.14 nM g^{-1} DW). However, it should be noted that these data were not replicated.
251 Even though these stresses increased IAA levels, the IAA-Asp levels only increased slightly likely due to the short
252 exposure time. If after the cold treatment, the anthers were kept on osmotic shock medium for four days, the IAA
253 concentration increased due to the presence of the exogenous IAA. However, the IAA-Asp concentrations showed
254 extremely large spikes with more than 1,000 nM g^{-1} DW. This amount is about 30 times more than that measured in
255 pea and some of this excess auxin was stored in form of IAA-Ala in an accessible form or further metabolized to
256 IAA-Glu.

257 The major bioactive forms of cytokinin detected in chickpea control anthers were tZR, cZR and iPA with
258 levels of 1.2, 0.8, and 0.4 nM g^{-1} DW, respectively. Only cZR increased after cold stress albeit not significantly
259 (Table 3). The medium contained 1.86 μM kinetin during the centrifugation, electroporation, and osmotic shock
260 treatments. Centrifugation and electroporation of anthers led to a temporary increase in cZR (2.9 and 3.1 nM g^{-1} DW
261 and iPA (0.9 nM g^{-1} DW) but not in tZR (0.6 and 0.5 nM g^{-1} DW). In contrast, cytokinin content of anthers exposed
262 to osmotic stress declined significantly; tZ, tZR, and dhZ were no longer detected and cZR, 2iP, iPA and dhZR
263 levels were very low even though the exposure to kinetin was over a 4-day period (Table 3).

264 Similar to field pea, none of the biologically active forms of gibberellin were detected in chickpea in
265 control anthers or after any of the stress treatments (Table 4). However, the GA_1 precursors GA_{53}, GA_{20}, GA_{19}, and
266 GA_9 of the 13-hydroxylation pathway were present as well as the catabolite GA_{29}. GA content peaked in control
267 anthers with 1.8 nM GA_8 and 2.6 nM g^{-1} DW GA_{20} whereas GA_{19} and GA_{29} increased slightly after the cold
268 treatment. The osmotic stress treatment completely eliminated this hormone with the exception of GA_{19} which was
269 still present in very small amounts (Table 4).

270

271 **Lentil** (*Lens culinaris Medik.*) var. CDC Maxim

272 Lentil treatments were cold storage of buds for 3 or 7 days, osmotic shock of anthers for 3 days at 4°C,
273 electroporation of anthers followed by osmotic shock for 3 days at 4°C, and centrifugation of anthers followed by
274 osmotic shock for 3 days at 4°C. No growth regulators were applied during stress treatments. Control anthers
275 contained 1.1 nM g^{-1}DW ABA. The lowest ABA concentration was observed in the 7d cold treatment with 0.4 nM
276 g^{-1} DW and the highest level of more than 1.3 nM g^{-1} DW after the osmotic shock treatments (Table 1). This is in
277 contrast to pea and chickpea where osmotic shock resulted in the lowest ABA levels. Control anthers contained high
278 levels of PA (182.6 nM g^{-1} DW) similar to pea var. Frisson. The 3-day cold treatment about halved this amount but
279 the lowest levels were achieved in the 7-day cold treatment. DPA concentrations peaked in anthers from the 3-day
280 cold treatment followed by the control. However, the osmotic shock treatments in conjunction with cold could not
281 reduce ABA or PA and DPA concentrations to the levels in the other two pulses (Table 1).

282 The IAA content of control anthers was 1.0 nM g^{-1} DW and increased to 3.7 nM after the 7-day cold
283 treatment (Table 2). This concentration was higher than the IAA content for var. Frisson but lower than the
284 maximum value for the cold +C treatment of var. CDC April. The major difference between lentil and the other two
285 pulses was in the IAA-Asp content. IAA-Asp only reached a maximum concentration of 1.3 nM g^{-1} DW in the
286 control and the 7-day cold treatment (Table 2) whereas the other species achieved much higher values of more than
287 40 nM for pea and 1,200 nM g^{-1} DW for chickpea.

288 Similar to pea and chickpea, the major cytokinins in lentil anthers were cZR, 2iP, and iPA with the
289 highest concentrations in the 3-day cold treatment and in the control (Table 3). The osmotic shock treatments

290 reduced cytokinin content to a level similar to that of pea and chickpea.

291 Eight different kinds of gibberellins were detected in lentil anthers but similar to pea and chickpea, no 292 bioactive forms were found (Table 4). The control anthers contained the highest amounts of GA_{29} and GA_8 which 293 are GA_1 catabolites. GA_{44} and GA_{19}, both precursors of GA_1, were also detected. The 7d cold and the osmotic shock 294 treatments reduced GA content to low levels (Table 4) similar to pea and chickpea.

295 Androgenesis inducing stress treatments caused an increase in the proportional levels of active auxins as 296 compared to all other hormones measured in all species tested. Specifically, osmotic shock is causing the 297 proportional increase in IAA for Frisson, while it required a pyramiding of stress treatments and osmotic shock to 298 achieve the same proportions in CDC April and CDC Xena (data not shown). This holds true for CDC Maxim 299 except the total amount of proportional difference is 2-3 times lower and it appears to be caused by 7 days at 4^0C. 300 CDC April has a greater amount of IAA/ABA than all other pulses tested when pyramiding treatments. Although 301 the spike in active auxin to ABA levels is the greatest in CDC April, this trend is seen in all other genotypes and 302 indicates that auxin may be more important than ABA for triggering androgenesis in legumes.

303

304 **Discussion**

305 Our investigation showed that androgenesis in legumes is mediated via phytohormones and that auxin 306 played a major role in this process after application of different stresses. Auxin is involved in many plant growth 307 functions including flowering and fertilization (Aloni et al., 2006; Hirano et al., 2008; Tromas et al., 2010; Zhao, 308 2010). In pea, IAA levels spiked after centrifugation in var. CDC April to about five times the concentration of the 309 control anthers (Table 2). In var. Frisson, IAA levels merely doubled in all stress treatments compared to the control 310 with a slightly higher response after sonication. However, osmotic shock in combination with the other treatments 311 (cold+C+E+S+O) reduced IAA levels considerably. In chickpea, IAA levels increased after centrifugation, 312 electroporation, and osmotic shock compare to the control or cold treatment but this was likely the result of the 313 exogenous IAA in the medium. In lentil, the IAA concentration after 7 days of cold was similar to levels in pea var. 314 Frisson after cold plus sonication (Table 2). Tanaka (1973) was the first to report that centrifugation of tobacco 315 anthers in the presence of IAA improved androgenesis induction. In contrast, Sangwan-Norreel (1977) found a

316 positive effect of centrifugation without the addition of hormones to *Datura innoxia* anthers. The requirement for
317 exogenous auxin during centrifugation seems to be species-dependent.

318 The major hormone in the two responsive legumes was the IAA conjugate, IAA-Asp. Anthers of both pea
319 and especially chickpea contained extremely high concentrations of IAA-Asp after cold, centrifugation,
320 electroporation or sonication stress in pea of up to 46 nM g^{-1} DW or after osmotic shock in chickpea of up to 1,284
321 nM g^{-1} DW (Table 2). In contrast, the recalcitrant lentil had no such peaks and the maximum concentration of IAA-
322 Asp was less than 1.3 nM g^{-1} DW (Table 2). This huge concentration difference might be related to the lack of
323 response of lentil to androgenesis induction. Whether the increase in IAA-Asp seen in microspores of legumes is
324 due to an increased breakdown of IAA or whether it is due to an increased need for IAA-Asp for some unknown
325 function is unclear. Most authors consider IAA-Asp a degradation product of IAA and if there is an increase in the
326 metabolites going through the tryptophan-dependent pathway, there will be a corresponding increase in IAA
327 degradation products (Normanly et al., 1995). Potentially, the various stresses applied to pea and chickpea caused
328 IAA spikes which quickly degrade causing IAA-Asp spikes. In contrast, several authors have suggested that IAA-
329 Asp may be biologically active and its function more complex than merely a mechanism for removal of free IAA
330 (Böttcher et al., 2010; Oetiker and Aeschbacher, 1997). The GH3 protein catalyzes the conjugation of IAA to IAA-
331 Asp. GH3-mediated auxin homeostasis may have a role in a plant's response to stress and be necessary for re-
332 allocation of resources for survival (Ghanashyam and Jain 2009, Park et al., 2007; Simon and Petrášek, 2011).
333 Ostrowski and Jakubowska (2011) found this enzyme to be most active in the early stages of pea seed development
334 (5 mm stage), implying a potential need for IAA-Asp. High concentrations of IAA-Asp (3,231 pM g^{-1} FW) have
335 been found in mature anthers of rice (Hirano et al., 2008), connected to ripening in grape and tomato and have been
336 linked to temperature sensitivity in cell cultures of henbane (Böttcher et al., 2010; Oetiker and Aeschbacher, 1997).
337 Similarly, Sasaki et al. (1994) showed that embryogenic cells of carrot not only have 15 times more IAA, but also an
338 increase in IAA-Asp, whereas the non-embryogenic cells had Ox-IAA-Asp (oxindole-3-acetylaspartic acid), a
339 potential degradation product of IAA-Asp.

340 The chlorinated auxin, 4-Cl-IAA, could not be investigated in this analysis and IAA-Asp production in
341 field pea could also be linked to this potent hormone which is present in pea fruits with a unique mode of action
342 (Engvild et al., 1981; Park et al., 2010). Its biosynthesis is independent of IAA and has a higher activity in pea,

343 which may be linked to increased stability (DeMason and Polowick, 2009, Simon and Petrásek, 2011). 4-Cl-IAA
344 may also be present in lentil but has not been reported in chickpea (Engvild et al., 1981; Reinecke, 1999).

345 The concentration of ABA and its catabolites spiked in control and cold-treated anthers and decreased after
346 osmotic shock treatment in the three legume species investigated (Table 1). Similarly, cold pre-treatment was found
347 beneficial for barley (*Hordeum vulgare* L.), tobacco (*Nicotiana tabacum* L) or triticale (x*Triticosecale*) androgenesis
348 due to improved microspore viability and increased response (Imamura and Harada, 1980; van Bergen et al., 1999,
349 Wang et al., 1999, Żur et al. 2008). However, pyramiding the other stresses such as centrifugation or electroporation
350 on top of the cold stress did not increase ABA levels indicating that there was no additive effect from the different
351 treatments. In contrast, osmotic shock treatment reduced ABA and catabolite content to very low levels especially in
352 the combined treatment cold +C+E+S+O in pea and cold +C+E+O in chickpea.

353 The predominant cytokinin was cZR in all three legumes but chickpea also responded with higher iPA
354 concentrations whereas both pea varieties showed increases in 2iP after some stresses (Table 3). Reports on
355 embryogenesis found that the *cis*-isomers predominated during pea and chickpea seed development especially
356 during the heart-shaped embryo stage (Emery et al., 1998; Quesnelle and Emery, 2007). No information is available
357 on the active cytokinin forms in lentil. Trans-isomers are thought to be biologically more active (Sakakibara, 2006;
358 Werner and Schmülling, 2009). However, Quesnelle and Emery (2007) showed that pea embryos could grow
359 equally well with either *cis*- or *trans*- zeatin indicating that cZ or its riboside are active cytokinins in this species. In
360 pea and chickpea, the highest cZR concentration occurred after application of the centrifugation and electroporation
361 stresses but in control and 3d cold-treated lentil anthers. Tret'yakova et al. (2009) found that the total cytokinin
362 content increased almost two fold from 284 to 1,324 ng g^{-1} DW in androgenetic callus of larch (*Larix sibirica*
363 Ledeb.). In our experiments, osmotic shock treatment reduced cytokinin content to very low levels (Table 3). Sáenz
364 et al. (2010) reported that the total cytokinin content in coconut (*Cococ nucifera* L.) was lower in embryogenic
365 compare to non-embryogenic callus. Hence, it seems that the effect of the osmotic shock treatment in reducing
366 hormone content might be of significance in the induction process. Exogenously supplied kinetin was present during
367 the 4-day osmotic shock treatment of chickpea anthers but this did not result in increased cytokinin levels (Table 3)
368 even though this hormone is rapidly metabolized (Sakakibara, 2006).

369 Although gibberellins are required for normal pollen, anther and seed development, a complete

370 understanding of their specific function remains unknown (Swain and Singh, 2005). In our study, no bioactive GAs
371 were found not even in the control anthers (Table 4). It is feasible that we collected anthers at a stage when GA
372 content started to drop off or that there was a dilution effect of the anther cells on the measurement of GA levels in
373 the microspores. However, it is more likely that we interrupted the formation of GAs, hence the increase in
374 precursors, by triggering stress reactions. Despite the need for this hormone immediately after fertilization (Alabadi
375 et al., 2009; Dorcey et al., 2009), GAs suppress the embryogenic LEC transcription factor cascade which initiates
376 embryogenesis. It is not surprising that stresses which trigger androgenesis would decrease its presence. A plant's
377 gibberellin levels are particularly controlled by the biosynthesis enzymes GA_{20}-oxidase and GA_3-oxidase and the
378 catabolism enzyme GA_{20}-oxidase (Fleet and Sun, 2005). It could be that the stress treatments which induce
379 androgenesis were suppressing the conversion of GA_{19} to GA_{20} or the production of the GA_{20}-oxidase enzyme.
380 However, this enzyme is also important in the formation of GA_{19} from GA_{53} and GA_{44}. A similar effect was reported
381 in wheat tissues where the GA_{19} intermediate had an especially low affinity for the GA_{20}-oxidase enzyme and
382 therefore tended to accumulate (Appleford et al., 2006).

383 Osmotic shock treatment seems to be different from the other stresses because this treatment reduced the
384 content of all hormones with the exception of IAA and IAA-Asp in chickpea. The high concentration of IAA-Glu in
385 chickpea indicates that the IAA concentration in the medium could be too high or the exposure to this hormone too
386 long. Since a short period of low hormone content seems to be a necessary transition phase required for the switch
387 from pollen development to embryo induction in pea, it would be interesting to determine if a reduced IAA
388 concentration in the medium or a secondary period of osmotic shock treatment without IAA would improve
389 induction in this species.

390 Androgenesis induction was successful for pea and chickpea but not for lentil. Our data showed that this
391 might be linked to auxin with a possible involvement of IAA-Asp. The ratio between auxin and ABA also indicates
392 that androgenesis in legumes has a greater auxin component than other species especially cereals, where increased
393 androgenesis and somatic embryogenesis was related to increased ABA levels (Wang et al., 1999; Żur et al., 2008).
394 We are clearly only at the beginning of understanding what governs androgenesis induction in legumes.

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546

547 Table 1. Content of ABA and its metabolites in nM g^{-1} DW in anthers after cold, centrifugation (C), electroporation (E), sonication (S), or osmotic shock (O).
548 Data presented are means ±SE and n indicates number of replicates per treatment. ND not detected

Species & Cultivar	Treatments	n	ABA	*t*-ABA	DPA	PA	neo-PA	ABA-GE	7'OH-ABA
Pisum	Control	2	2.14±0.03	1.55±0.07	131.03±9.78	34.57± 6.73	0.62±0.00	0.14±0.01	0.12±0.02
CDC	Cold	1	0.51	1.40	41.08	173.19	0.14	ND	ND
April	Cold +C	2	0.46±0.02	2.65±0.12	13.04±2.06	1.65± 0.96	0.10±0.01	ND	ND
	Cold +E	2	0.37±0.00	0.80±0.06	15.19±0.98	1.04± 0.02	0.10±0.00	0.09±0.01	0.03±0.03
	Cold +S	2	0.23±0.00	0.54±0.04	15.52±0.30	2.37± 1.10	0.06±0.01	0.20±0.05	ND
	Cold +C+E	2	0.21±0.05	1.04±0.13	12.46±1.83	19.39±18.63	0.02±0.02	0.10±0.01	ND
	Cold +C+E+S	2	0.24±0.04	0.90±0.16	11.51±0.82	9.72± 8.98	0.09±0.04	0.05±0.05	ND
	Cold +O	3	0.24±0.02	0.17±0.02	0.36±0.05	0.17± 0.02		0.37±0.00	ND
	Cold+C+E+S+O	1	ND	0.03	0.03	ND	ND	0.02	ND
Pisum	Control	2	2.61±0.16	0.72±0.00	35.13±1.96	189.10±71.57	0.41±0.00	0.07±0.02	0.02±0.02
Frisson	Cold	1	0.37	1.61	38.11	33.81	0.16	0.05	ND
	Cold +E	2	0.32±0.00	0.97±0.02	22.39±0.88	18.71± 0.95	0.05±0.01	0.08±0.01	ND
	Cold +C	3	0.30±0.01	1.16±0.03	51.18±3.15	14.62± 3.34	0.12±0.00	0.06±0.03	0.02±0.02
	Cold +S	2	0.29±0.02	0.94±0.04	28.28±1.51	16.13± 2.69	0.07±0.00	0.09±0.00	ND
	Cold +C+E	3	0.54±0.01	0.90±0.04	24.93±1.41	5.53± 0.80	0.14±0.02	0.15±0.01	0.02±0.02
	Cold +C+E+S	3	0.27±0.00	1.21±0.05	43.63±2.63	9.05± 1.15	0.10±0.00	0.02±0.02	ND
	Cold +O	2	0.07±0.01	0.06±0.03	0.05±0.05	0.12± 0.00	ND	0.16±0.03	ND
	Cold+C+E+S+O	2	0.09±0.03	0.01±0.01	0.11±0.03	0.14± 0.02	ND	0.19±0.02	0.01±0.01
Cicer	Control	4	10.38±0.98	1.19±0.13	4.32±0.77	26.12± 6.12	0.40±0.11	0.53±0.26	0.33±0.17
CDC	Cold	3	5.11±0.66	1.10±0.12	6.28±1.15	21.27± 5.61	0.53±0.05	0.62±0.18	0.43±0.04
Xena	Cold +C	2	2.02±0.95	1.10±0.04	12.22±0.51	112.12±41.43	0.38±0.05	0.18±0.02	0.17±0.05
	Cold +E	2	2.43±0.77	1.13±0.35	13.80±4.00	105.74±44.31	0.48±0.01	0.18±0.05	0.24±0.03
	Cold +E+O	3	0.68±0.21	0.12±0.01	1.19±0.17	1.34± 0.27	0.02±0.01	0.58±0.19	0.06±0.03
	Cold +C+O	3	0.48±0.12	0.06±0.03	1.02±0.09	1.41± 0.11	0.03±0.02	0.54±0.14	0.06±0.03
	Cold +O	3	1.45±0.30	0.19±0.05	1.48±0.54	1.80± 0.43	0.05±0.01	1.38±0.15	0.18±0.03

	Cold+C+E+O	3	0.41±0.09	0.09±0.00	1.03±0.19	1.17± 0.11	0.03±0.01	0.70±0.30	0.05±0.02
Lens	Control	1	1.10	0.86	22.47	182.58	0.28	ND	0.46
CDC	3 d Cold	1	1.15	1.26	27.25	87.25	0.24	ND	0.87
Maxim	7d Cold	2	0.43±0.05	1.12±0.12	4.36±0.53	30.14±19.39	0.10±0.01	ND	0.48±0.05
	O+ 3d Cold	2	1.49±0.01	0.20±0.03	12.00±0.72	26.14 ± 0.99	0.05±0.01	0.04±0.04	0.39±0.06
	E+O+3d Cold	2	1.34±0.06	0.19±0.07	6.14±1.45	19.73 ± 0.90	0.08±0.00	0.05±0.05	0.41±0.06
	C+O+3d Cold	2	1.53±0.04	0.17±0.05	9.76±0.03	20.17 ± 0.03	0.05±0.01	0.12±0.01	0.53±0.08

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550 Table 2. Content of auxin and its metabolites in nM g^{-1} DW in anthers after cold, centrifugation (C), electroporation (E), sonication (S), or osmotic shock (O).
551 Data presented are means ±SE and n indicates number of replicates per treatment. ND not detected

Species & Cultivar	Treatments	n	IAA	IAA-Asp	IAA-Glu	IAA-Ala
Pisum	Control	2	1.61±0.06	11.40±0.20	0.41±0.03	ND
CDC	Cold	1	1.62	40.4	0.46	ND
April	Cold +C	2	8.91±1.43	29.83±2.10	0.34±0.03	ND
	Cold +E	2	1.73±0.32	34.13±5.41	0.39±0.03	ND
	Cold +S	2	2.32±0.48	29.73±3.57	0.37±0.05	ND
	Cold +C+E	2	3.48±1.53	42.70±7.95	0.50±0.01	ND
	Cold +C+E+S	2	2.82±0.99	38.83±7.10	0.50±0.12	ND
	Cold +O	3	1.49±0.36	17.89±0.88	0.28±0.01	ND
	Cold +C+E+S+O	1	1.01	3.98	0.26	ND
Pisum	Control	2	0.87±0.02	3.12±0.40	0.17±0.00	ND
Frisson	Cold	1	1.77	36.57	0.47	ND
	Cold +C	3	2.47±0.27	42.51±3.62	0.56±0.02	ND
	Cold +E	2	2.34±0.44	45.74±3.42	0.52±0.00	ND
	Cold +S	2	2.76±0.31	44.79±2.58	0.62±0.02	ND
	Cold +C+E	3	2.24±0.31	41.88±3.99	0.46±0.06	ND
	Cold +C+E+S	3	2.05±0.05	39.40±1.73	0.52±0.01	ND

	Cold +O	2	1.76±0.18	6.09±0.49	0.13±0.00	ND
	Cold +C+E+S+O	2	1.45±0.32	5.45±0.93	0.04±0.04	ND
Cicer	Control	4	0.61±0.07	0.07±0.02	ND	ND
CDC	Cold	3	0.42±0.04	0.04±0.04	ND	ND
Xena	Cold +C –IAA	1	0.43	0.04	ND	ND
	Cold +C +IAA	1	5.57	1.45	0.04	ND
	Cold +E –IAA	1	0.14	0.07	ND	ND
	Cold +E+IAA	1	3.37	0.64	ND	ND
	Cold+O	3	3.30±0.62	1,219.12±155.30	39.44± 06.30	0.14±0.07
	Cold +C+O	3	5.10±2.88	1,284.24±226.47	60.77±12.27	0.35±0.17
	Cold +E+O	3	2.04±0.18	971.13±310.39	33.17±06.47	0.15±0.03
	Cold +C+E+O		2.63±1.18	1,022.88±322.39	35.94±14.25	0.08±0.05
Lens	Control	1	1.02	1.29	0.03	ND
CDC	3 d Cold	1	1.26	0.95	ND	ND
Maxim	7d Cold	2	3.07±0.39	1.25±0.15	0.01± 0.01	ND
	O+ 3d Cold	2	1.00±0.12	0.66±0.08	0.01± 0.01	ND
	E+O+3d Cold	2	0.82±0.18	0.62±0.06	ND	ND
	C+O+3d Cold	2	0.78±0.03	0.66±0.05	ND	ND

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553 Table 3. Content of cytokinin and its metabolites in nM g^{-1} DW in anthers after cold, centrifugation (C), electroporation (E), sonication (S), or osmotic shock (O).
554 Data presented are means ±SE and n indicates number of replicates per treatment. ND not detected

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Species & Cultivar	Treatments	n	c-ZR	2iP	iPA	c-Z	t-Z	t-ZR	dhZ	dh-ZR
Pisum	Control[2]	2	0.58±0.01	0.87±0.03	0.20±0.01	0.05±0.00	0.01±0.01	0.05±0.00	ND	ND
CDC	Cold	1	0.9	0.67	0.1	0.01	ND	ND	ND	ND
April	Cold +C	2	1.84±0.01	0.53±0.03	0.10±0.00	ND	ND	ND	ND	ND
	Cold +E	2	0.62±0.02	0.50±0.02	0.08±0.00	0.01±0.01	ND	ND	ND	ND
	Cold +S	2	0.36±0.01	0.63±0.01	0.05±0.00	0.01±0.01	ND	ND	ND	ND

	Cold +C+E[2]	2	0.86±0.09	0.67±0.07	0.09±0.00	0.01±0.01	ND	ND	ND	ND
	Cold +C+E+S	2	0.81±0.06	0.65±0.07	0.10±0.01	ND	ND	ND	ND	ND
	Cold +O	3	0.14±0.00	0.04±0.00	0.04±0.01	ND	0.01±0.01	ND	0.03±0.01	ND
	Cold+C+E+S+O	1	0.02	0.08	0.02	0.02	ND	ND	ND	ND
Pisum	Control	2	0.16±0.01	0.82±0.03	0.26±0.06	0.03±0.00	ND	ND	ND	ND
Frisson	Cold	1	1.15	0.87	0.2	ND	ND	ND	ND	ND
	Cold +C	3	1.04±0.05	0.80±0.03	0.14±0.00	ND	ND	ND	ND	ND
	Cold +E	2	1.09±0.05	0.88±0.01	0.14±0.00	0.01±0.01	ND	ND	ND	ND
	Cold +S	2	1.17±0.08	0.88±0.03	0.16±0.01	ND	ND	ND	ND	ND
	Cold +C+E	3	0.67±0.00	0.68±0.03	0.09±0.01	ND	ND	ND	ND	ND
	Cold +C+E+S	3	1.06±0.03	0.82±0.02	0.16±0.00	ND	ND	ND	ND	ND
	Cold +O	2	0.06±0.04	0.01±0.01	0.01±0.01	ND	ND	ND	ND	ND
	Cold+C+E+S+O	2	0.01±0.01	0.01±0.01	0.01±0.01	ND	ND	ND	ND	ND
Cicer	Control	4	0.80±0.22	0.04±0.05	0.39±0.12	ND	0.25±0.04	1.18±0.26	0.16±0.04	0.27±0.08
CDC	Cold	3	1.02±0.29	ND	0.23±0.02	ND	0.12±0.01	1.08±0.12	0.10±0.02	0.47±0.05
Xena	Cold +C	2	2.86±1.06	0.05±0.01	0.94±0.33	ND	0.15±0.00	0.60±0.01	0.15±0.02	0.39±0.03
	Cold +E	2	3.14±0.54	0.06±0.03	0.88±0.17	ND	0.12±0.02	0.46±0.05	0.12±0.02	0.28±0.09
	Cold +C+O	3	0.12±0.05	ND	ND	ND	ND	ND	ND	0.02±0.02
	Cold +E+O	3	0.12±0.06	0.02±0.02	ND	ND	ND	ND	ND	0.01±0.01
	Cold +O	3	0.15±0.07	ND	ND	ND	ND	ND	ND	0.00±0.00
	Cold+C+E+O	3	0.13±0.05	ND	ND	ND	ND	ND	ND	0.01±0.01
Lens	Control	1	1.04	0.35	0.1	ND	ND	ND	ND	ND
CDC	3 d Cold	1	1.16	0.33	0.18	ND	ND	ND	ND	ND
Maxim	7d Cold	1	0.25±0.01	0.24±0.01	0.01±0.01	ND	ND	ND	ND	ND
	O+ 3d Cold	2	0.41±0.01	0.16±0.00	0.04±0.00	ND	ND	ND	ND	ND
	E+O+3d Cold	2	0.32±0.02	0.12±0.00	0.03±0.00	ND	ND	ND	ND	ND
	C+O+3d Cold	2	0.15±0.02	0.14±0.01	0.02±0.00	ND	ND	ND	ND	ND

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559 Table 4. Content of gibberellin and its metabolites in nM g^{-1} DW in anthers after cold, centrifugation (C), electroporation (E), sonication (S), or osmotic shock
560 (O). Data presented are means ±SE and n indicates number of replicates per treatment. ND not detected

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Species & Cultivar	Treatments	n	GA_{19}	GA_{20}	GA_8	GA_9	GA_{29}	GA_{44}	GA_{53}
Pisum	Control	2	0.81±0.01	3.41±0.06	ND	0.66±0.05	ND	0.12±0.03	0.08±0.01
CDC	Cold	1	0.99	0.25	ND	ND	ND	ND	ND
April	Cold +C	2	1.63±0.05	ND	ND	ND	ND	ND	ND
	Cold +E	2	1.04±0.02	0.11±0.01	ND	ND	ND	ND	ND
	Cold +S	2	1.98±0.06	ND	ND	ND	ND	ND	0.11±0.01
	Cold +C+E	2	1.14±0.14	ND	ND	ND	ND	ND	0.02±0.02
	Cold +C+E+S	2	1.34±0.25	ND	ND	ND	ND	ND	0.08±0.05
	Cold +O	3	0.37±0.02	ND	ND	ND	ND	ND	ND
	Cold +C+E+S+O	1	0.05	ND	ND	ND	ND	ND	ND
Pisum	Control	2	0.40±0.01	2.31±0.08	ND	3.77±0.20	2.89±2.89	0.10±0.00	0.01±0.01
Frisson	Cold	1	1.93	0.11	ND	ND	ND	ND	0.17
	Cold +C	3	1.00±0.03	ND	ND	ND	ND	ND	0.06±0.01
	Cold +E	2	1.82±0.08	0.04±0.04	ND	ND	ND	ND	0.08±0.01
	Cold +S	2	1.38±0.13	ND	ND	ND	ND	ND	0.06±0.00
	Cold +C+E	3	1.12±0.01	ND	ND	ND	ND	ND	0.01±0.01
	Cold +C+E+S	3	1.07±0.06	0.03±0.03	ND	ND	ND	ND	0.02±0.02
	Cold +O	2	0.06±0.00	ND	ND	ND	ND	ND	ND
	Cold +C+E+S+O	2	0.04±0.04	ND	ND	ND	ND	ND	ND
Cicer	Control	4	0.28±0.06	2.62±0.51	1.75±0.32	0.09±0.07	0.26±0.20	ND	0.03±0.02
CDC	Cold	3	0.75±0.18	1.17±0.24	1.13±0.09	ND	0.71±0.36	ND	0.18±0.06
Xena	Cold +C	2	0.72±0.16	1.17±0.50	0.87±0.12	ND	ND	ND	0.20±0.01
	Cold +E	2	0.95±0.07	0.93±0.61	0.73±0.30	ND	ND	ND	0.14±0.04
	Cold +C+O	3	0.07±0.02	ND	ND	ND	ND	ND	ND
	Cold +E+O	3	0.08±0.04	ND	ND	ND	ND	ND	ND
	Cold +O	3	0.02±0.02	ND	ND	ND	ND	ND	ND
	Cold +C+E+O	3	0.07±0.04	ND	ND	ND	ND	ND	ND

Lens	Control	1	0.48	0.5	0.94	ND	1.48	1.39	0.15
CDC	3 d Cold	1	0.47	ND	0.32	ND	ND	0.26	0.27
Maxim	7d Cold	2	0.26±0.03	ND	ND	ND	ND	ND	0.04±0.04
	O+3d Cold	2	0.40±0.03	ND	0.25±0.00	ND	ND	0.30±0.01	0.11±0.02
	E+O + 3d Cold	2	0.18±0.06	ND	ND	ND	ND	0.05±0.05	0.02±0.02
	C+O + 3d Cold	2	0.10±0.06	ND	ND	ND	ND	0.04±0.04	ND

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