1 **Increased illumination levels enhance biosynthesis of aloenin A and aloin B in *Aloe arborescens* Mill., but lower their per-plant yield.**

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11

12 **Abstract:** Leaves of *Aloe arborescens* Mill. are a relevant source of secondary metabolites of

13 pharmaceutical relevance. Notwithstanding, specialized cultivations of *A. arborescens* are still

14 rather limited, and a straightforward agronomical research addressed to the obtainment of high-

15 quality material is lacking. With the purpose to fill this gap, from 2016 to 2018, a trial was arranged

16 to evaluate the growth and development of *A. arborescens*, along with the production of four

17 active metabolites (aloin A and B, aloenin, and isoaloeresin A) with varying some growth

18 conditions. Two growth substrates ("A"- a commercial substrate, and "B"- the same substrate +

19 20% perlite), two durations of pre-transplant open-air storage ("stress", 7 and 14 days), and 3

20 illumination levels obtained by means of shadow nets with different mesh dimensions (SL: full

21 sunlight; T50: 50% shading; T70: 70% shading) were tested, combined in a factorial experimental

22 layout with 3 repetitions. In general, light intensity was the most crucial experimental factor,

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whereas the effects of growth substrate and pre-transplant stress were scarce and limited in time. The addition of perlite to the growth substrate gave the best results in terms of leaf size and root growth, without any significant effect on the yield of active metabolites. The increasing shading level caused a parallel increase of several biometrical characters of plants (height, number of leaves per plant and mean diameter of the stem), whereas the number of suckers per plant was positively affected by the increase of illumination level. The illumination level was also responsible for significant variations in the content of all secondary metabolites, except for aloin A, that resulted statistically not different among the illumination treatments (from 0.80 to 0.98 mg 100 mg^{-1}). The content of aloenin A and aloin B was higher in the plants exposed in full sun, statistically well differentiated from those exposed to 70% shadow (2.0 vs. 1.4 mg 100 mg^{-1} aloenin A, and 1.12 vs 0.86 mg 100 mg^{-1} aloin B, in full sun and 70% shadow, respectively). Contrastingly, the highest levels of isoaloeresin D were reached in the two shaded treatments (4.98 mg 100 mg^{-1} in 50% shadow and 4.89 mg 100 mg^{-1} in 70% shadow), whereas the full sun treatment reached the lowest value. The higher number of leaves in plants exposed at reduced illumination, however, brought to increased total amounts per plant of aloin (A and B) and aloenin A with increasing shadow levels.

Keywords: Aloe arborescens, cultivation, shading, secondary metabolites, aloin, aloenin

Highlights

- *A. arborescens* was cultivated at three increasing shading levels
- Plants height, leaves/plant, stem diameter increased with shading
- Percentages of aloenin A and aloin B were higher in full sunlight.
- Content per plant of all studied metabolites was lower in full sunlight.

46 **1. Introduction**

47 *Aloe arborescens* Mill. (fam. Asphodelaceae, subfam. Asphodeloideae, tribe Aloeae, gen Aloe)
48 (Smith et al., 2012; Chase et al., 2016) is a poliannual succulent flowering plant, native to tropical
49 and southern Africa, which grows spontaneous in many areas of the world. *A. arborescens* has a
50 rather large development (according to Smith et al., 2012, it grows high up to 5 meters). The plant
51 may be branched, with alternate long spiny leaves. Leaves are greyish-green and can reach 50-60
52 cm in length, and grow in dense rosettes at the apex of the branches. The inflorescences (racemes)
53 are distinctly inverted-cone shaped, and the flowers are pencil-shaped and quite long. Flowering
54 begins on mature plants (2-3 years old). The flower color ranges from scarlet to yellow, through
55 many hues of red, orange, light salmon-pink, and apricot (Smith et al. 2008).

56 *A. arborescens* is a well-known CAM species (Kluge et al., 1979). In these plants, atmospheric
57 CO_2 is fixed to PEP in the dark, with production of oxalacetate and, through subsequent reduction,
58 malate. In the light, stored malic acid is decarboxylated again, generating CO_2 that is
59 photosynthetically converted into carbohydrates (Denius and Homann, 1972; Liu et al., 2018). Since
60 the stomata of CAM plants open at night and close during the day, all gas exchanges occur at night;
61 this helps prevent excessive water loss and keeps moisture within the tissues. As a consequence,
62 water use efficiency in this type of plants may be five times greater than the C_3 or three times than
63 the C_4 plants (Rodríguez-García et al., 2007; Silva et al., 2010). Because of this feature, together
64 with the water leaf reservoir, *A. arborescens* is generally recognized as a xerophytic species (Liu et
65 al., 2011), and thrives in warm and semi-arid environments.

66 All plants belonging to the genus *Aloe* are endowed with various amounts of a gel, contained in
67 in a parenchymatic leaf section (often termed hydrenchyma), that is located between upper and
68 lower mesophyll. It is a mucilage, formed by 96% water, and for the remaining part by a complex

69 mixture of vitamins (A, B, C, E), sugars, lignin, saponins, minerals, aminoacids and enzymes (Park et
70 al., 1998; Dagne et al. 2000).
71 *A. vera* is one of the *Aloe* species in which this mucilage is more abundant, and this is probably
72 one of the main reasons of the large success of this species as a pharmaceutical ingredient (Smith
73 et al., 2008; Ahlawat and Khatkar, 2011; Grace et al., 2015). The gel obtained from *A. vera* is
74 traditionally addressed to external use as a wound-healing, tissue regenerating and detergent item
75 (WHO, 1999).
76 *A. arborescens,* whose leaves are generally less thick and dealing with a lower gel content, has a
77 lower interest than *A. vera* for mucilages extraction. Hence, although in recent times some interest
78 has arisen towards gel from *A. arborescens* to prepare edible coatings able to prolong shelf-life in
79 peach and plum fruits (Guillén et al., 2013), the real allure of *A. arborescens* relies on different
80 reasons.
81 The species acquired worldwide popularity in the late 1990's, after the diffusion of a preparation
82 from *A. arborescens* leaves, reputed to have significant healing properties for many tumoral forms.
83 The so-termed "Father Zago's recipe", named after the Brazilian Franciscan friar who reported to
84 have reproduced its preparation from a Brazilian popular remedy, is obtained by grinding and
85 mixing the entire *A. arborescens* leaf, further mixed with spirit and, probably also because of its
86 remarkably bitter taste, with raw honey (Zago, 1997).
87 Much research has been conducted all over the world to explore the antitumor potentialities of
88 these preparations (Tsuda et al., 1993; Shimpo et al., 2001; Lissoni et al., 2009; Harlev et al., 2012)
89 and, in general, the therapeutic actions attributed to *A. arborescens.* Besides exhibiting larger
90 amounts of total polyphenols with respect to other *Aloe* species (Cardarelli et al., 2017), the leaves
91 of *A. arborescens* contain a great deal of active molecules, including complex sugars (among which,

acemannan), anthraquinones, lignin, saponins, and others (Cardarelli et al., 2017; Nazeam et al., 2017).

Among these metabolites, aloin A (barbaloin) and aloin B (isobarbaloin) are two diastereoisomers of the glycoside 10-β-D-glucopiranosil-aloe-emodina anthrone, whose mixture is often collectively termed aloin or barbaloin (Sharma et al., 2014). Aloin is the bitter principle of *Aloe* juice, and, although it has been retrieved in no fewer than 68 different *Aloe* species, it is one of the most important components of *A. arborescens* extract, where it is generally present in higher amount than in *A. vera* (0.6 vs 0.3% approx., according to Li et al., 2003; about 0.2% in both species, according to Lucini et al., 2013). Aloin A and, at a lesser extent, aloenin, exhibited significant anti-inflammatory activity (Gutterman and Chauser-Volfson, 2000, 2000a). In *A. arborescens*, it mostly accumulates in the large parenchymatous cells of vascular bundles, in the vascular bundle sheath and in the aquiferous tissue sheath, being practically absent from the inner leaf parenchyma (Li et al., 2003).

Aloenin A and B are instead two glycosidic derivatives, a monoglycosyde the former and a diglycoside the latter. Both compounds are usually more easily detectable in *A. arborescens* than Aloin A and B; they were found in about the same quantity as the total of Aloin A and B (Kuzuya et al., 2001; Beppu et al., 2004).

Isoaloeresin D is a cinnamoyl-chromone with recognized anti-inflammatory properties. Although some authors (Beppu et al., 2004) claim that it is a characteristic phenolic compound of *A. vera*, it was not always detected in all experiments concerning this species (Saccù et al., 2001), otherwise being represented in other *Aloe* species such as *A. nyeriensis* (Cardarelli et al., 2017).

Within the genus *Aloe*, those active metabolites are distributed differently according to plant's characteristics (taxon, plant age, leaf fractions) (Lucini et al., 2015), but also in relation to external

115 factors, such as environmental conditions, cropping techniques, storage and marketing methods
116 (Cardarelli et al., 2017).

117 In leaves of *A. arborescens*, the relative amounts of aloin A, aloenin and aloeresin were found to
118 increase from the basis to the apex, and from the abaxial (lower) to the adaxial (upper) side, with
119 negligible variations among differently-oriented leaves (Gutterman and Chauser-Volfson, 2000,
120 2000a). The content in those metabolites also varied as a consequence of temperature, being higher
121 in spring and summer (April to September) than in winter (October to March) (Beppu et al., 2004).

122 Differently to *A. vera*, specialized cultivations of *A. arborescens*, addressed to the obtainment of
123 valuable pharmaceutical and industrial raw matter, are rather limited. A survey of the available
124 literature shows that many aspects A*. arborescens* cultivation are still to be deepened. Commonly,
125 the same cropping techniques applied to *A. vera* are also used for *A. arborescens*, without a specific
126 validation of the different crop characteristics. Much research is necessary to verify many aspects
127 of cropping technique suitable for *A. arborescens*, above all as concerns the effect of the major
128 agricultural practices on the production and storage of active metabolites. Among the cropping
129 techniques to study, propagation management and light exposure deserve a major interest.

130 Both *A. arborescens* and *A. vera* are propagated vegetatively, by means of suckers (offsprings)
131 and rhizome cuttings (Cristiano et al. 2016); *A. arborescens* deals with 3-4 suckers per plant almost
132 twice in one year, a much more abundant production of suckers than *A. vera*. A short storage
133 period of suckers (5-10 days) before transplant is usually advised, in order to lower moisture for
134 preventing diseases, and with the goal to facilitate roots growth and promote root suberisation
135 (Cristiano et al., 2016). As far, no information is available about the actual advantage offered by
136 this technique, nor about its effects, if any, on the content of active metabolites.

137 Scarce information is also available about the more suitable growth substrate for *A. arborescens*
138 cultivation. In *A. vera*, the adoption of an organic substrate is generally suggested (Cristiano et al.,
139 2016; Das and Chattopadhay, 2004), with the best results, in terms of yield and size of leaves,
140 obtained with a 50:50 soil:manure growth substrate (Hasanuzzaman et al., 2008). No
141 straightforward research has been conducted on the evaluation of the effects exerted by the
142 growth substrate on the active metabolites content in *Aloe arborescens*.

143 Although the metabolic pathways leading to the biosynthesis of many secondary metabolites are
144 still a matter of debate (Sangwan et al., 2001; Korulkin and Muzychkina, 2014), due to their
145 dependence upon photosynthetic process, it may be reasonably supposed that their production
146 and accumulation may be modified by light exposure. Indeed, many experiments on medicinal and
147 aromatic plants have demonstrated that this effect occurs, although generalization is difficult
148 because of the high variability of plants and metabolites of interest (Hälvä et al., 1992; Sangwan et
149 al., 2001). In *A. vera*, photosynthetic efficacy was found to decrease with increasing light
150 intensities, and this effect was more pronounced in conditions of water deficit stress (Hazrati et al.,
151 2016). Despite its importance, however, also the effect of illumination intensity on *A. arborescens*
152 has not been targeted to many studies. A comparison between aloin (aloin A + aloin B) content in
153 3-year-old plants of *A. arborescens* and *A. vera*, submitted to contrasting illumination levels, was
154 discussed by Lucini et al. (2013). In their experiment, aloin content (expressed as g kg^{-1} fresh
155 weight) in *A. arborescens* leaves was found to decrease with lowering light intensity. More research
156 was otherwise dedicated to *A. vera.* Paez and coworkers (2000) exposed *A. vera* plants to three
157 irradiance levels, namely: full sunlight, partial (30% full sunlight), and deep shade (10% full
158 sunlight), and evaluated the effect exerted on plant biomass and main metabolites content. The
159 authors concluded that the highest illumination level allowed the best growth conditions on dry

160 weight of leaves, roots and stems, as well as on the number of axillary buds. Contrastingly, plants
161 exposed to partial shade had a higher number of longer – albeit not wider - leaves. No significant
162 effect was detected on the aloin content in leaf exudate. An inhibitory effect of shading on aloin
163 content in *A. vera* was otherwise reported by Li et al. (2006), who found that this effect was
164 significantly higher in younger than in older leaves: in young leaves, aloin content under shading
165 was 63.33 % higher than under natural light, whereas in older leaves grown under the same
166 conditions, aloin content was only 23.77% higher.

167 Hence, there is room for research aimed to deepen those aspects of cultivation technique in *A.*
168 *arborescens,* with the goal to contribute in pointing out a straightforward cropping management
169 for industrial purpose. This research was aimed to study the growth and development, as well as
170 the production of some secondary metabolites (Aloin A and B, aloenin, and isoaloeresin A) with
171 varying the growth substrate, the duration of pre-transplant stress, and the illumination intensity.
172

173 **2. Material and methods**

174 *2.1 Experimental layout and measurements on plants*

175 2.1.1 Plants arrangement and management

176 The trial was arranged in 2016, using 144 suckers of *A. arborescens*, kindly gifted by the
177 Cooperative Company “Le Shiare” from Marsala (TP, Sicily). Rooting offsets were homogeneous
178 both in size and in development (5 cm length from the bottom to the apex, and about 6 leaves
179 each), and they were collected on 29 June 2016 from mother plants at least 2-year-old and in
180 excellent phytosanitary condition. After collection, offsprings were transferred to the CREA-DC
181 facilities in Bagheria (PA, Sicily) for the transplant. One half of the rooting offsets (i.e. 72
182 individuals) were exposed to open air in the shadow for 7 days (T7), whereas in the remaining part

183 this stress period was prolonged for an additional week (T14). The definitive positioning of the
184 rooting offsets in the pots was therefore arranged in two different moments: on 6 July 2016 (after
185 one week of pre-transplant stress – T7) and 14 July 2016 (after 2 weeks of pre-transplant stress –
186 T14).

187 On each transplanting date, rooting offsets were inserted in two different growth substrates,
188 thereafter termed A and B. Substrate A was the commercial substrate "Vigorplant Terriccio
189 Cinquestelle®" (Vigorplant Italia srl), that is a mixture of sphagnum peat, graded volcanic rock and
190 bentonite clay; pH 5.5 – 6.5, whereas substrate B was obtained by mixing the same substrate (80%)
191 with 20% expanded perlite. Both substrates were mixed with 1.5 kg m^{-3} of NPK Original Gold
192 (Compo Expert, Italy), a compound inorganic fertilizer containing 15% inorganic N, 9% P_2O_5, and
193 15% K_2O.

194 At the end of these operations, all pots were watered to saturation point, and submitted to
195 three different shading levels. In detail, one third of the plants (48 individuals) were positioned in
196 full sun (direct sunlight; SL). Irradiance levels measured in sunny days from May to August by
197 means of a LiCor Li-250A radiometer (LiCor, Lincoln, NE) ranged between 1134 and 2024 mmol m^{-2}
198 s^{-1}; this value was assumed to correspond to the maximum light amount received by plant canopy
199 in the full sunlight treatment. Other two groups of plants, including 48 individuals each, were
200 allocated inside two separated tunnels, in which different shading levels (50% and 70%, termed T50
201 and T70, respectively) were obtained by means of two black shadow nets with different mesh
202 dimensions.

203 Each elementary experimental unit was therefore formed by 4 plants, arranged in a factorial
204 layout with three repetitions.

205 After transplant, plants were periodically surveyed in order to evaluate their growth and
206 phytosanitary conditions. A supplementary fertilization was applied in May 2017, distributing 15 g
207 of NPK compound fertilizer in each pot. In summer months, pots were watered every week to
208 saturation point. To avoid overcrowding within the pots, at the end of March and in the first days
209 of December 2018, all suckers were completely removed.

210

211 2.1.2 Measurements on leaves

212 Throughout the first cultivation season (from late summer to autumn 2016), the main traits of
213 leaves morphology were studied, by measuring leaf length (from the bottom to the tip), leaf basal
214 width, and leaf basal thickness (figure 1). Such measurements were taken on 23 September, 6
215 October, 19 October, 11 November and 10 December, on two different sets of leaves, chosen in
216 the lower and in the upper parts of the same plants (i.e. among the oldest and the younger leaves,
217 respectively); the three upper leaves were always excluded from measurements, as generally they
218 were not fully developed.

219

220 2.1.3 Biometrical non-destructive measurements

221 In 2017 and 2018, as soon as plants had completed their first year after transplant, the major
222 biometrical non-destructive observations were made. These measurements were taken about
223 every month, and included: plant height, diameter of main stem, number of offspring (if present),
224 number of fully developed leaves per plant (also in this case, excluding the three youngest upper
225 leaves).

226

227 2.1.4 Destructive measurements

228 On 31 October 2017, i.e. at the end of the vegetative season of the second year, on a random
229 sample of 3 plants for each experimental group (i.e. 108 plants in total), the destructive
230 observations were made. Hence, every newly-formed sprout was detached from the main stem
231 and weighed. After offspring removal, each plant was gently removed from pot and weighed, so
232 obtaining the fresh weight (g) of both its epigeal and (after rinsing with water to eliminate the soil
233 residuals) hypogeal part. All fully developed leaves (i.e. excluding the three small immature leaves
234 in upper position) were cut from the stem and quickly inserted in a graduate Becker, in order to
235 allow latex to percolate. After about 15 minutes, leaves were singularly weighed. A 3-leaves sample
236 per each experimental unit was taken, including three leaves from each plant, free from visible
237 damage or disease, taken from the lower, medium and upper section, respectively. On this 3-leaves
238 sample, the weight incidence of the three major components was taken: thorns (from the leaf
239 border, considered unsuitable for pharmaceutical purposes); hydrocolloid (gel); epidermidis. Small
240 samples of each fraction were put in stove at 105°C for 24 h, in order to determine their moisture
241 content, further reported in percent.
242
243 2.1.5 Sample preparation for chemical analysis
244 Since preliminary observations showed that the main secondary metabolites of interest were
245 absent in the gel, being rather represented in the latex, samplings were made with the goal to
246 avoid latex dispersal from leaves. Hence, from each experimental unit, three leaves per plant were
247 picked up from the upper, medium and lower part of the plant, respectively. The leaves were
248 weighed and chopped into smaller parts, immediately sealed into Falcon probes (50 mL each),
249 lyophilized and delivered to the labs of CNR-ICB in Catania (CT-Sicily) for further analyses.

250 Later on, 500 mg of lyophilized plant material were manually cut in small pieces (about 5 mm^2)
251 and extracted in 8 mL glass vials for 24 h with MeOH under continuous shaking. The resulting light
252 green colored suspensions were filtered on PTFE 0.45 μ filters (PALL Corporation), put into 2 mL
253 amber vials and sent to analytical determinations.
254
255 *2.2 HPLC/DAD/MS quantitative analyses*
256 Quantitative analyses on the metabolites of interest (aloin A and B, aloenin, and isoaloeresin)
257 were carried out on an Ultimate3000 instrument equipped with a binary high-pressure pump, a
258 Photodiode Array Detector (Thermo Scientific, Italy). Chromatographic runs were all performed
259 using a reverse-phase column (Gemini C_{18}, 250 x 4.6 mm, 5 µm particle size, Phenomenex, Italy).
260 Chromatographic runs were carried out following the already published method of Park et al.
261 (1998) with slight modifications as follows. A gradient of B (MeOH) in A (Water), 0 min: 25% B; 5
262 min: 30% B; 15 min: 35% B; 50 min: 70% B; 60 min: 70% B; 65 min: 25% B; then kept for 5 min at
263 25% B. The solvent flow rate was 0.7 mL/min. Pure standards of Aloenin A, Aloin A, Aloin B,
264 Isoaloeresin were purchased from Labochem Science SRL (Catania, Italy).
265 Quantifications were carried out, using the corresponding reference substances, at 293 nm for
266 aloenin A (r^2 = 0.9997), aloin A (r^2 = 0.9999); aloin B (r^2 = 0.9995) and isoaloeresin (r^2 = 0.9893). In
267 order to confirm peak assignments, a series of HPLC/ESI/MS analyses were performed on a
268 significant number of samples. The HPLC apparatus used was the same described above, as per as
269 chromatographic column and elution program, whilst ESI mass spectra were acquired by a Thermo
270 Scientific Exactive Plu Orbitra MS (Thermo Fisher Scientific, Inc., Milan, Italy), using a heated
271 electrospray ionization (HESI II) interface. Mass spectra were recorded operating in negative ion

272 mode as already reported (Napoli et al., 2018). Data acquisition and analyses were performed using
273 the Excalibur software.
274
275 *2.3 Statistical treatment of data*
276 All data were submitted to statistical analysis according to the chosen experimental design
277 (factorial with three replications), by means of the software Minitab® v 17.1.0 (Minitab Inc., State
278 College, PA, USA, 2013). Data obtained from all surveys were submitted to a preliminary ANOVA
279 including all experimental factors, namely: Illumination (I), Substrate (Sb), Stress (s), and whenever
280 measurements had been repeated in time, also the observation date (D). The General Linear Model
281 procedure *(y=f(x))* was applied; all measurements on plants were considered as dependent
282 variables *(y)*, and all experimental factors were set as independent variables (*x;* fixed factors).
283 Whenever the ANOVA offered statistically significant results, the differences among mean values
284 were appreciated through Tukey's test at $P \leq 0.05$ (Gomez and Gomez, 1984).
285
286 **3. Results and discussion**
287 *3.1. Growth and development of plants*
288 The ANOVA carried out on the major growth and development parameters measured on *A.*
289 *arborescens* on the first date after transplant (September 2016) and in the first October decade in
290 the first, second and third trial year (table 1) enlightened that contrasting illumination levels were
291 always responsible for variations in all the measured parameters. No significant differences were
292 otherwise found due to the "stress" effect, neither alone nor in interaction with other factors,
293 whereas the effect "substrate" was found significant only in the first two surveys (September and
294 October 2016) of number of leaves per plant. A significant second level interaction (I x Sb x s) was

295 only found in the ANOVA for the first survey date (September 2016) in the number of suckers per
296 plant.
297 In all plants (starting from initial values of about 5 cm), height values increased very quickly from
298 the beginning of the experiment (figure 2), and after 2 years from transplant, plants reached about
299 8-9 folds their initial value. Wide differences showed up among treatments, and plants growing in
300 direct sunlight (SL) were always significantly shorter than plants growing under shading mesh. In
301 the last survey of the third year (October 2018), although this general trend was unvaried, the first
302 two treatments (SL and T50) took statistically similar mean values (31.2 and 32.7 cm, respectively),
303 whereas the most shadowed treatment (T70), with 37.4 cm, was well distinguished from the other
304 two.
305 The number of leaves per plant (figure 3) markedly increased in the first trial year, from 5-6 in
306 the rooted young plants at the start of cultivation to 10-12 at the end of 2016. In the following
307 years, an increase in the number of leaves was still evident, although lower than in 2016, and in the
308 last survey (October 2018), plants reached about 13.5 (SL), 14.3 (T50) and 16.2 (T70) leaves per
309 each. As recorded for plant heights, also in this case the plants growing in full sunlight exhibited a
310 sharply lower number of leaves per plant than the other two treatments. In the SL treatment,
311 furthermore, a decrease in the number of leaves per plant was recorded from January to April
312 (2017) and from March to May (2018), due to the wilting and falling of the lower leaves. In the T50
313 and T70 treatments, this phenomenon was much less evident, and plants simply reduced the
314 emission rate of new leaves in the same periods. The ANOVA carried out on this variable (table 1)
315 showed significant differences due to the substrate in the first two surveys; although plants grown
316 in the substrate A had always a slightly higher number of leaves than in substrate B (data not
317 shown), this difference was statistically significant only in the two first surveys (19 September 2016

and 10 October 2016) when the number of leaves per plant reached 7.6 (A) vs. 7.1 (B), and 9.2 (A) vs. 9.8 (B) in the two dates, respectively. No effect was detected due to the variation of pre-transplant stress duration (s), neither due to all second- and third-order interactions.

The measured diameter of stem (figure 4) increased linearly throughout the trial, from less than 1 cm in all treatments, to 1.5-1.6 cm in the first days of October 2018. Significant differences appeared sometimes at ANOVA in the diverse surveys, showing the most relevant increase in the plants growing under the highest shading degree (T70), which kept the highest measured values along all the second and third trial year. In the last survey (October 2018), all treatments exhibited similar diameter values.

The number of suckers per plant (figure 5) counted throughout the trial (August 2016 to December 2018) showed a sharp increase, from about 1 sucker/plant at the end of 2016 to about 2.5 at the end of all surveys. At ANOVA (table 1) significant differences showed up only about the different illumination levels and, according to the substrate type, only in the first survey (September 2016). No significant difference was found related to the contrasting stress level. Plants started forming offspring very early. In the first year, more suckers were counted on T50 treatment, whereas in the second year the offspring emission slowed down and kept stable until the end of the year. In the second year, the SL treatments started increasing, and the rank SL>T50>T70 started to delineate. This difference remained after the first suckers removal, and at the end of the third year it was even more marked. In 2018, plants exposed to full sunlight expressed the highest production of suckers (about 4 suckers/plant). Plants from the T50 treatment kept a rather constant number of suckers from January 2017 to April 2017, with an increase in the following months until December 2018 (about 2 suckers/plant). Plants from treatment T70 always showed a lower number of suckers/plant compared with the other two treatments.

341

342 ### *3.2 Shape, size, and structure of leaves*

343 In their first growth period, from September to November 2016, leaves of *A. arborescens*
344 evidenced a marked increase in all development parameters (length, basal width and basal
345 thickness; figure 6), showing in time a 27% length increase (from 15.3 to 19.4 cm), a 32% increase
346 in basal width (from 1.5 to 1.9 cm) and a 46% increase in basal thickness (from 0.4 to 0.6 cm). The
347 ANOVA (table 2) showed that these values were significantly affected by all the tested variability
348 factors, with the exception of the stress duration (s), that only showed some effect in interaction
349 with other factors. Smaller leaves were found in the plants exposed to full sun, and in general, in
350 plants grown on the "A" substrate. A significant difference was also found between leaves in the
351 lower and upper position in the same plant. Lower leaves (L) were generally smaller (shorter, less
352 wide and less thick) than those growing in the upper part of plants, although this difference was
353 less evident in the last survey.

354 Consistently with this trend, the data obtained from the destructive leaf measurements carried
355 out about one year later confirmed the crucial importance of the illumination level, as well as the
356 loss of significant effect exerted by the different substrates (Sb) and stress conditions (s). The
357 results of ANOVA carried out on the fresh and dry weight of one single leaf and its fractions,
358 according to the experimental factors (table 3) show significant differences among illumination
359 treatments for the values of fresh weight of one leaf, that resulted totally unaffected by the other
360 experimental factors, either alone or in combination.

361 Fresh weight of one leaf (Figure 7a) significantly increased from 13.2 to 20.8 g with increasing
362 shading, being spikes (2.3, 3.6, and 4.1 g, in SL, T50, and T70, respectively) and parenchymatic inner
363 tissue (4.9, 7.2 and 8.7 g), the most affected plant fractions. The most relevant fraction of fresh

364 weight of one leaf was the inner parenchyma in the T70 treatment (41.6%), whereas in the more
365 illuminated plants (SL and T50) it was the outer epidermis (45.8% and 40.2%, respectively). Those
366 differences were slightly less evident in the measured values of dry weight (figure 7b). Leaf dry
367 weight was on average 3.3-3.7 g, without significant differences among treatments. Among leaf
368 components, statistically significant differences show up only in plants grown at full exposition,
369 where spikes (1.1 g, corresponding to the 31.8% of leaf dry weight) and inner parenchyma (0.9 g,
370 corresponding to the 26.2%) reached the lowest values.
371

372 *3.3 Partitioning of plant biomass*

373 All measurements taken on plant biomass were affected by illumination level (table 4 and figure
374 8), whereas the type of substrate exerted significant influence only on the mass of roots, and the
375 duration of pre-transplant stress revealed a significant effect only in association with illumination
376 level (I x s interaction), and only on roots. Total plant biomass (aerial parts + roots) under the
377 highest shading conditions (T70) reached an average value of 426.0 g/plant, a 68% higher value
378 than in plants growing in full light (254.1 g/plant). In all experimental conditions, leaves
379 represented the highest percentage of plant biomass (47.5, 49.8, and 54.8% in the SL, T50, and T70
380 treatments, respectively). The stem (48.5 g/plant in SL, 63.0 in T50, and 79.7 in T70) formed,
381 instead, a lower percentage of plant biomass (from 17.0%, T50, to 18.7%, T70, and 19.1%, SL). The
382 mass of roots was negatively affected by illumination, since it reached the lowest value in the SL
383 treatment (85.0 g/plant, corresponding to the 33.4% of total plant biomass). Hence, the best
384 growing conditions, identified as the highest shading level (T70), not only allowed a higher biomass
385 production, but also a comparatively higher production of stems and roots. In a broad sense, these
386 results are similar to those obtained by Cardarelli et al. (2013), who tested the response of *A.*

387 *arborescens* plants to contrasting levels of organic fertilizers, finding in the highly fertilized plants a
388 higher biomass value, composed by a proportionally slightly lower fraction of leaves, with a higher
389 incidence of stems and roots.

390 Besides illumination, also substrate induced variations in roots biomass (table 4; figure 9): the
391 substrate B allowed, on average, the best growth of roots (124.7 g/plant), whereas in the substrate
392 A root biomass reached 89.3 g/plant. An improved root growth due to the addition of perlite to the
393 cultivation substrate has been already observed in other plant species (Fascella et al., 2008;
394 Fascella and Zizzo, 2009; Fascella et al., 2012). Being a highly porous material, perlite allows a more
395 adequate equilibrium between air and moisture content of the growing mixture and, consequently,
396 a more balanced composition of substrate B with respect to substrate A.

397 Likewise, the mean values of the "I x s" interaction (Figure 9) exhibited a $P \leq 0.01$ significance at
398 ANOVA (table 4). A 14-day pre-transplant storage seemed allowing a better roots growth, that,
399 conversely, was reduced in absence of this pre-treatment. Hence, the best conditions for roots
400 growth were obtained with a more prolonged stress period before transplant, exception made for
401 the T70 treatment, where however plant metabolism was addressed towards the production of a
402 higher aerial biomass.

403

404 *3.4 Secondary metabolites*

405 The results of ANOVA by illumination level (I), type of substrate (Sb) and duration of pre-
406 transplant stress (s), on the average content of each studied active metabolites (expressed as a
407 percentage of dry plant material), as well as on the estimated content in one single leaf (mg $leaf^{-1}$)
408 and in one single plant (mg $plant^{-1}$) are reported in table 5. The correspondent means, averaged by

409 illumination level, are shown in the graphs in figure 10.

410 The illumination level was responsible for significant variations in the percentage content of all
411 secondary metabolites, except for aloin A, that took values around 0.8-1.0%, resulting unaffected
412 by the illumination level. The type of substrate did not evidence any isolated effect (main effect of
413 Sb always not significant), whereas in some cases it acted in combination with illumination, but
414 always enhancing the effect of the former. Similarly, the main effect of the duration of pre-
415 transplant stress (s) was never statistically significant, exception made for the percentage of aloin
416 B, where the effect was however exerted in association with the illumination level (I).

417 The average content in aloenin A and aloin B was higher in the plants exposed in full sun (SL),
418 statistically well differentiated from those exposed to 70% shadow (2.0 vs. 1.4% aloenin A, and 1.12
419 vs 0.86% aloin B, in SL and T70 treatments, respectively). The trend of isoaloeresin D was different,
420 since this compound reached the highest levels (4.98 and 4.89%, statistically not different) in the
421 two shadowed treatments (T50 and T70), whereas the SL treatment reached the lowest value. The
422 percentage of aloin A resulted statistically not different among the different illumination
423 treatments (from 0.80% in T70 to 0.98% in SL). The remaining other two experimental factors
424 (substrate and stress duration) did not cause significant differences.

425 Similar results were obtained by Lucini et al (2013), who found that aloin content in plants of *A.*
426 *arborescens* linearly decreased as sunlight intensity was reduced to 70 and 40%. Plant reactivity to
427 illumination levels could explain the different amounts in selected secondary metabolites that was
428 assessed by Gutterman and Chauser-Volfson (2000) between adaxial and abaxial leaf sections,
429 being the adaxial (upper) part the one more exposed to light.

430 When the content in the active metabolites was reported to the whole leaf (figure 10), the
431 overall trend resembles that already described about the percentages, although showing some

difference in the most abundant compound (isoaloeresin D). In this case as well, the statistical analysis confirms the similarity between data obtained into the two shadowed treatments (T50 and T70). A higher difference shows up when the same determinations are expressed as a fraction of the entire plant (mg active metabolites/plant), i.e. taking into account all leaves per plant. As an example, the content in aloenin A in the three illumination levels follows an opposite trend than previously described. Hence, the lower number of leaves per plant observed in the plants exposed to full illumination allows a much lower yield of aloenin A per plant (111 mg) than the other two treatments (149 mg in T70 and 161 mg in T50). A similar feature can be observed in aloin A and B, whose content in mg per plant is opposite to that observed in the yield percentages. Otherwise, no difference could be detected in the content in isoaloeresin D.

4. Conclusions

This work allowed to point out some interesting features of *A. arborescens,* related to plant growth and development, as well as to the yield and storage of some secondary metabolites, as a function of light intensity, growth substrate and duration of pre-transplant stress. In general, light intensity was the most significant experimental factor, whereas growth substrate and pre-transplant stress, only in few cases exerted appreciable effects. The addition of perlite to the growth substrate gave the best results in terms of leaf size and root growth, without any significant effect on the yield of active metabolites. The increasing shading level (SL<T50<T70) caused a parallel increase of most biometrical characters of plants (height, number of leaves per plant and mean diameter of the stem), whereas the number of suckers per plant was positively affected by the increase of illumination level. When determined on one leaf-basis, yields of aloin (A and B) and aloenin A were more abundant in plants cultivated in full sun, whereas, on whole plant-basis, the

yields of the same compounds followed the trend SL<T70<T50. From the agronomical point of view, this statement is not without consequences, since it could be useful for *A. arborescens* growers to straightforward crop growth conditions according to the purpose of cultivation. A full-sun layout would probably be useful for nurseries and for supplying young plants, whereas for specialized cultivations of *A. arborescens*, addressed to pharmaceutical or cosmetic purposes, the adoption of 50% shading seems the most convenient condition, despite the comparatively lower unitary production of secondary metabolites.

These findings highlight for the first time the complex issue of cultivation of *Aloe arborescens* in view of phytochemicals production, and open new horizons to the industrial use of this species, which could represent a smart approach to increase the farmers' income.

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TABLES

Table 1. *Aloe arborescens*. Results of ANOVA (*F* values) for the major growth and development observations along the trial, according to illumination level (I), substrate (Sb) and duration of pre-transplant stress (s).

Source of variation	DF	plant height				leaves/plant				diameter of stem				suckers/plant			
		13-09-16	06-10-16	03-10-17	11-10-18	01-09-16	13-10-16	03-10-17	11-10-18	29-09-16	13-10-16	09-10-17	11-10-18	15-09-16	13-10-16	03-10-17	11-10-18
Illumination (I)	2	14.01***[a]	17.50***	36.32***	9.60**	22.27***	18.63***	63.39***	17.71***	2.47 ns	8.02**	10.03**	1.91 ns	5.02*	4.82*	4.00*	12.96***
Substrate (Sb)	1	3.64 ns	3.78 ns	1.97 ns	<1 ns	4.76*	6.12*	<1 ns	<1 ns	<1 ns	3.06 ns	<1 ns	<1 ns	<1 ns	1.50 ns	1.40 ns	<1 ns
Stress (s)	1	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	1.97 ns	5.83*	3.55 ns	<1 ns	<1 ns	2.02 ns	<1 ns	2.17 ns	<1 ns
I x Sb	2	<1 ns	1.11 ns	<1 ns	1.21 ns	1.37 ns	3.22 ns	<1 ns	<1 ns	1.31 ns	<1 ns	<1 ns	1.40 ns	1.07 ns	<1 ns	1.54 ns	1.50 ns
I x s	2	<1 ns	<1 ns	1.84 ns	<1 ns	<1 ns	<1 ns	1.82 ns	1.16 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns
Sb x s	1	2.91 ns	1.17 ns	<1 ns	<1 ns	3.53 ns	1.51 ns	<1 ns	1.46 ns	<1 ns	<1 ns	4.18 ns	2.46 ns	2.02 ns	2.08 ns	<1 ns	<1 ns
I x Sb x s	2	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	2.00 ns	<1 ns	1.40 ns	2.53 ns	<1 ns	<1 ns	2.77**	<1 ns	1.18 ns	1.80 ns
Error	24																
Total	35																

[a]results of ANOVA: *, significant at P≤0.05; **, significant at P ≤0.01; ***, significant at P≤0.001; n.s., non-significant.

Table 2. *Aloe arborescens*. Results of ANOVA (*F* values) for the measurements of leaf length, basal width, and basal thickness, according to date of observation (D), illumination level (I), substrate (Sb), duration of pre-transplant stress (s) and leaf position (LP).

Source of variation	DF	Leaf length	Leaf basal width	Leaf basal thickness
Date (D)	3	76.12***[a]	105.55***	75.30***
Illumination (I)	2	20.53***	26.14***	20.10***
Substrate (Sb)	1	18.12***	32.01***	11.06***
Stress (s)	1	3.11 ns	<1 ns	<1 ns
Leaf position (LP)	1	257.40***	215.93***	59.17***
Significant first order interactions				
D x I	6	<1 ns	2.69*	7.27***
D x Sb	3	<1 ns	3.75*	<1 ns
D x LP	3	47.18***	29.88***	10.60***
I x Sb	2	7.60**	10.44***	8.05***
Sb x s	1	4.90*	8.94**	1.42 ns
Significant second order interactions				
D x I x Sb	6	2.09 ns	1.85 ns	<1 ns
D x I x s	6	2.78*	1.12 ns	1.47 ns
D x Sb x LP	3	<1 ns	5.06**	<1 ns
D x s x LP	3	<1 ns	1.76 ns	<1 ns
I x Sb x s	2	<1 ns	5.76**	1.36 ns
I x Sb x LP	2	6.69**	4.41*	<1 ns
I x s x LP	2	3.17*	2.66 ns	2.77 ns
Significant third and fourth order interactions				
D x I x Sb x s	6	3.87***	2.13 ns	<1 ns
D x I x Sb x LP	6	1.32 ns	2.41*	<1 ns
D x I x s x LP	6	2.58*	1.29 ns	2.16*
I x Sb x s x LP	2	6.03**	11.61***	4.21*
D x I x Sb x s x LP	6	2.72*	2.12 ns	<1 ns
Error	168			
Total	263			

[a]results of ANOVA: *, significant at P≤0.05; **, significant at P ≤0.01; ***, significant at P≤0.001; n.s., non-significant.

609

Table 3. *Aloe arborescens*. Results of ANOVA (*F* values) for the fresh and dry weight of one entire leaf and its fractions (SP: spikes; EP: epidermis; G; gel), according to illumination level (I), substrate (Sb) and duration of pre-transplant stress (s).

Source of variation	DF	Fresh weight				Dry weight			
		Entire leaf	SP	EP	G	Entire leaf	SP	EP	G
Illumination (I)	2	17.83***[a]	56.22***	3.82 ns	22.19***	7.68**	11.14**	3.92*	74.16***
Substrate (Sb)	1	<1 ns	<1 ns	<1 ns	1.31 ns	5.89*	3.20 ns	3.03 ns	74.80***
Stress (s)	1	<1 ns	1.53 ns	<1 ns	<1 ns	1.82 ns	<1 ns	2.72 ns	<1 ns
I x Sb	2	<1 ns	3.67 ns	<1 ns	<1 ns	<1 ns	<1 ns	1.66 ns	11.56**
I x s	2	<1 ns	<1 ns	<1 ns	<1 ns	2.38 ns	<1 ns	2.29 ns	7.96**
Sb x s	1	<1 ns	<1 ns	<1 ns	<1 ns	1.11 ns	<1 ns	1.86 ns	<1 ns
I x Sb x s	2	2.33 ns	2.56 ns	1.48 ns	2.22 ns	1.85 ns	<1 ns	2.63 ns	1.99 ns
Error	12								
Total	23								

[a]results of ANOVA: *, significant at P≤0.05; **, significant at P ≤0.01; ***, significant at P≤0.001; n.s., non-significant.

610

Table 4. *Aloe arborescens*. Results of ANOVA (*F* values) for the measured mean fresh weight of one plant and its components, according to illumination level (I), substrate (Sb) and duration of pre-transplant stress (s).

Source of variation	DF	Entire plant	Aerial parts (leaves+stem)	Mature leaves	Stem	Roots
Illumination (I)	2	19.41***[a]	23.67***	24.16***	20.78***	8.39**
Substrate (Sb)	1	2.83 ns	<1 ns	<1 ns	2.47 ns	20.26***
Stress (s)	1	<1 ns	<1 ns	<1 ns	<1 ns	1.16 ns
I x Sb	2	<1 ns	<1 ns	<1 ns	<1 ns	2.51 ns
I x s	2	2.33 ns	<1 ns	<1 ns	<1 ns	7.15**
Sb x s	1	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns
I x Sb x s	2	2.29 ns	2.63 ns	2.42 ns	3.21 ns	1.02 ns
Error	12					
Total	23					

[a]results of ANOVA: *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; ***, significant at $P \leq 0.001$; n.s., non-significant.

Table 5. *Aloe arborescens*. Results of ANOVA (*F* values) for the content in active metabolites (Aloin A; Aloin B; Aloenin A; Isoaloeresin D), measured in percentage of d.m., in mg in the whole leaf, and in mg in the whole plant, according to illumination level (I), substrate (Sb) and duration of pre-transplant stress (s).

Source of variation	DF	Aloin A			Aloin B			Aloenin A			Isoaloeresin D		
		%	mg/leaf	mg/plant	%	mg/leaf	mg/plant	mg 100 mg^{-1} d.m.	mg/leaf	mg/plant	mg 100 mg^{-1} d.m.	mg/leaf	mg/plant
Illumination (I)	2	2.23 ns[a]	<1 ns	12.82***	3.69*	<1 ns	8.53**	6.90**	1.86 ns	8.79***	45.75***	40.86***	49.23***
Substrate (Sb)	1	<1 ns	1.36 ns	1.58 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns
Stress (s)	1	3.30 ns	5.26*	<1 ns	2.21 ns	3.50 ns	<1 ns	2.50 ns	4.22 ns	<1 ns	1.28 ns	2.57 ns	<1 ns
I x Sb	2	2.75 ns	6.03**	14.24***	3.25 ns	6.14**	11.91***	1.78 ns	4.25*	11.10***	<1 ns	<1 ns	1.81 ns
I x s	2	2.22 ns	1.37 ns	1.75 ns	4.41*	3.13 ns	2.91 ns	1.23 ns	<1 ns	<1 ns	1.29 ns	1.73 ns	<1 ns
Sb x s	1	1.15 ns	<1 ns	<1 ns	1.02 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns	<1 ns
I x Sb x s	2	<1 ns	1.63 ns	<1 ns	<1 ns	1.24 ns	<1 ns	1.40 ns	2.09 ns	<1 ns	<1 ns	<1 ns	<1 ns
Error	24												
Total	35												

[a]results of ANOVA: *, significant at P≤0.05; **, significant at P ≤0.01; ***, significant at P≤0.001; n.s., non-significant.

611 **Figure 1**. Measurements on *A. arborescens* leaves. 1: leaf length; 2: leaf width (basal); 3: leaf thickness
612 (basal).

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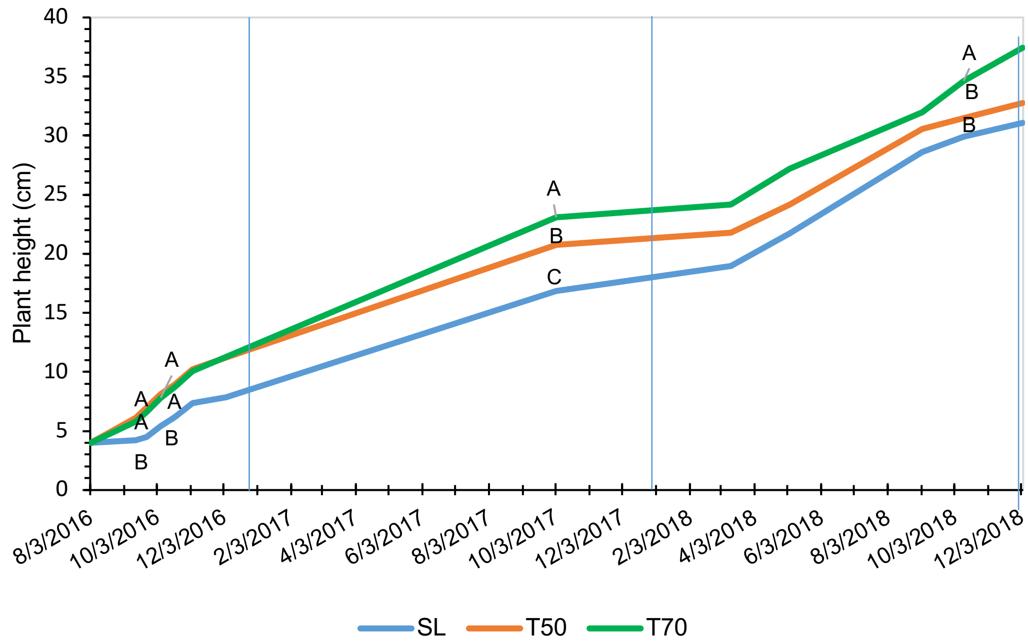

614
615 **Figure 2**. *Aloe arborescens.* Trend of plant height throughout the trial, across illumination intensity. For 4 selected dates,
616 different letters above each curve indicate a significant difference (P ≤ 0.05) among the means (Tukey's test).
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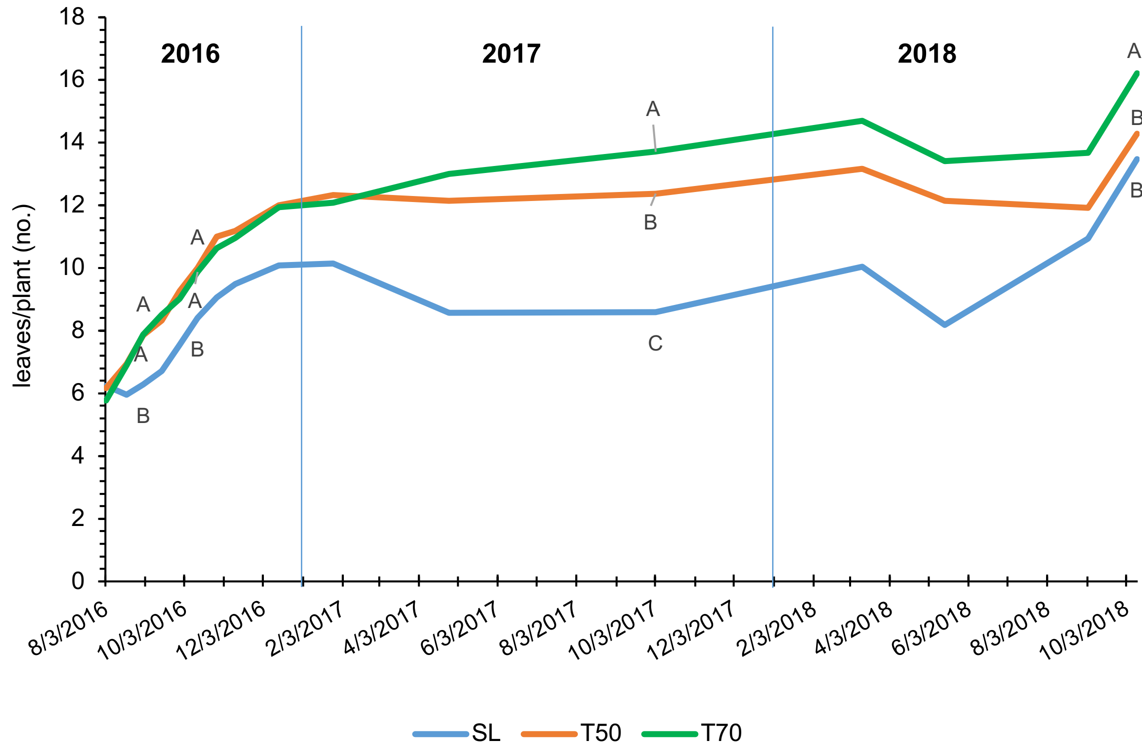

621
622 **Figure 3**. *Aloe arborescens*. Trend of number of leaves per plant throughout the trial, across illumination intensity. For 4
623 selected dates, different letters above each curve indicate a significant difference (P ≤ 0.05) among the means (Tukey's
624 test).
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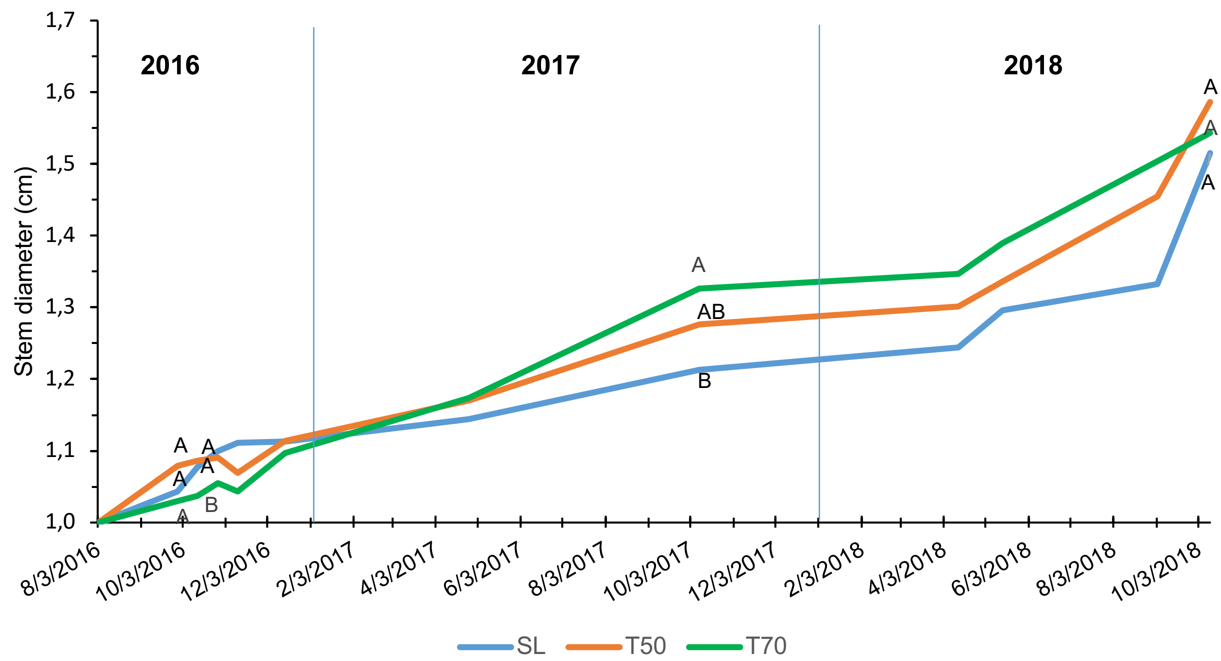

626
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628 **Figure 4**. *Aloe arborescens.* Trend of stem diameter throughout the trial, across illumination intensity. For 4 selected
629 dates, different letters above each curve indicate a significant difference ($P \leq 0.05$) among the means (Tukey's test).
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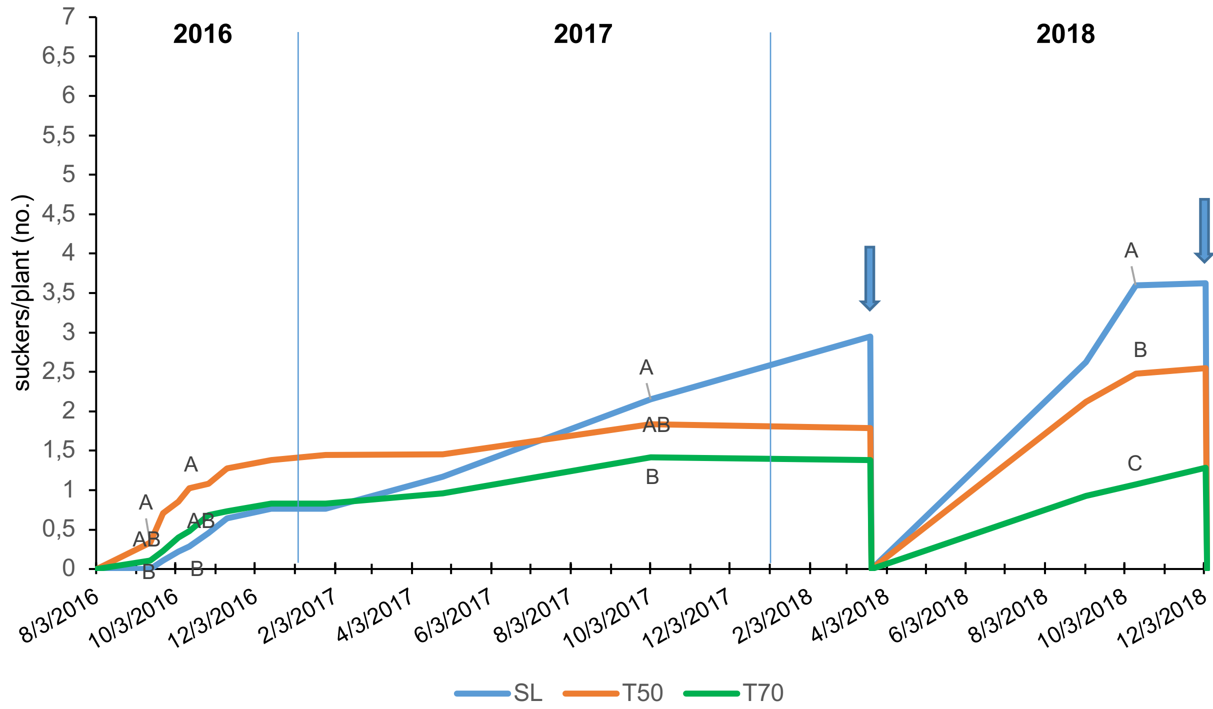

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635 **Figure 5**. *Aloe arborescens*. Trend of number of suckers/plant throughout the trial, across illumination intensity. Arrows
636 indicate the dates of complete suckers removal. For 4 selected dates, different letters above each curve indicate a
637 significant difference ($P \leq 0.05$) among the means (Tukey's test).
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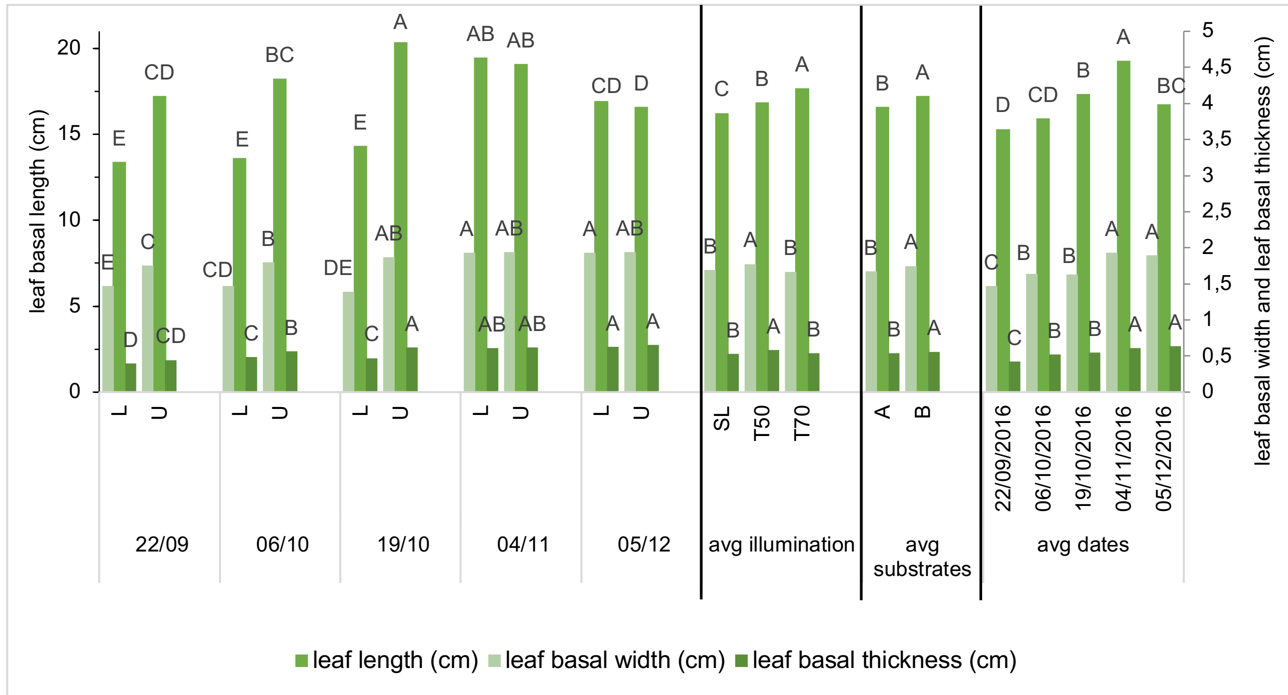

642
643
644 **Figure 6**. *Aloe arborescens*. Mean values of leaf basal width and thickness (left axis) and leaf length (right axis) in a factorial
645 experiment carried out in Bagheria (PA, Italy) in 2018; mean values of the interactions "date x position", and mean values
646 of the main factors "illumination", "substrate", and "date of measurement". For each variable and group, different letters
647 indicate a significant difference (P ≤ 0.05) among the means (Tukey's test). L: lower leaves, U: upper leaves; SL, T50, T70:
648 shading levels; A, B: substrates.
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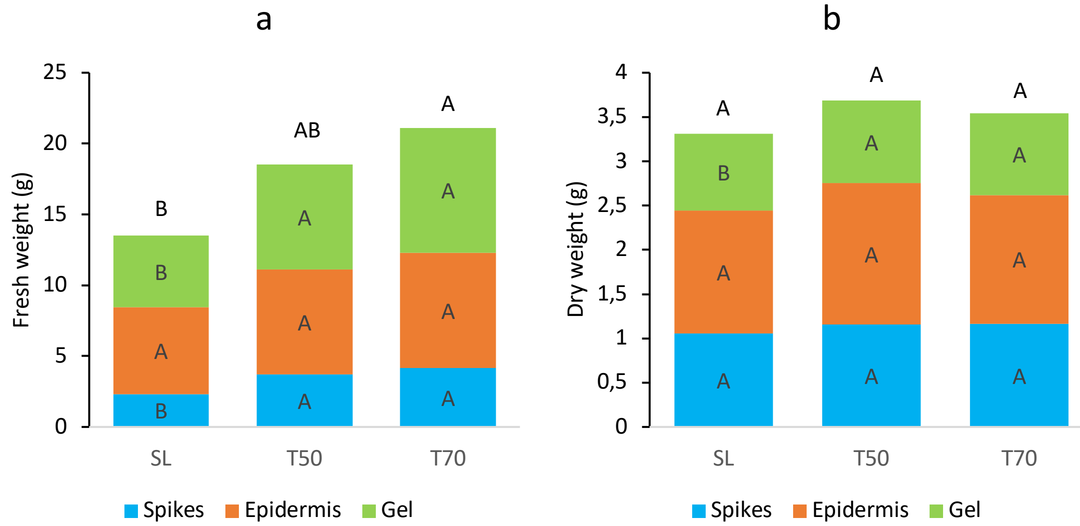

652

653 **Figure 7**. *Aloe arborescens*. Partitioning of fresh weight (a) and dry weight (b) of leaves in spikes, outer epidermis and
654 parenchymatic tissue (gel), with contrasting illumination intensities (SL= full sunlight; T50: 50% shading; T70: 70%
655 shading). Within each graph and leaf fraction, different letters indicate a significant difference (P ≤ 0.05) among the
656 illumination treatments; letters above the bars refer to the fresh and dry weight, respectively, of one entire leaf (Tukey's
657 test).
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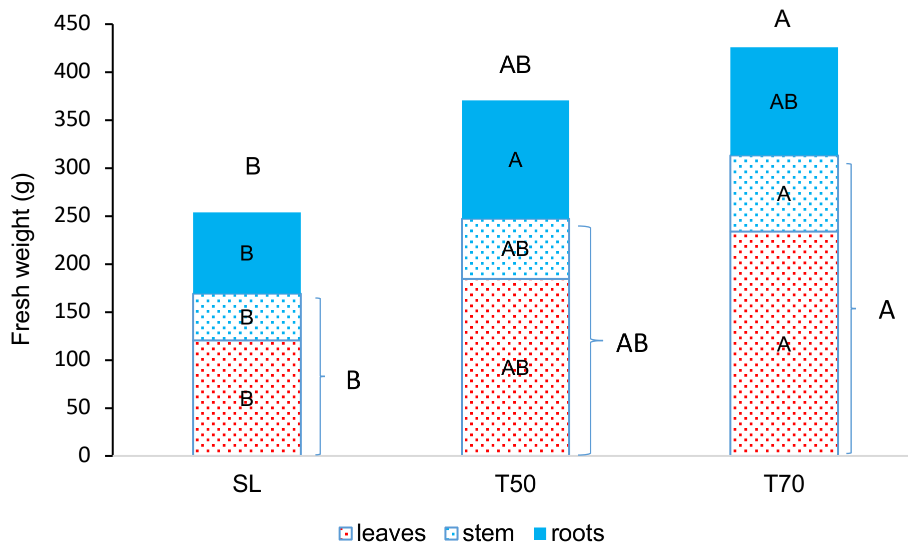

660
661
662 **Figure 8**. *Aloe arborescens.* Partitioning of the fresh weight of one plant (leaves, stem and roots), with contrasting
663 illumination intensities (SL= full sunlight; T50: 50% shading; T70: 70% shading). Within each plant fraction, different letters
664 indicate a significant difference ($P \leq 0.05$; Tukey's test) among the illumination treatments; letters above the bars refer to
665 the fresh weight of the entire plant; letters on the right side of each bar refer to the sum of aerial organs (leaves + stem).
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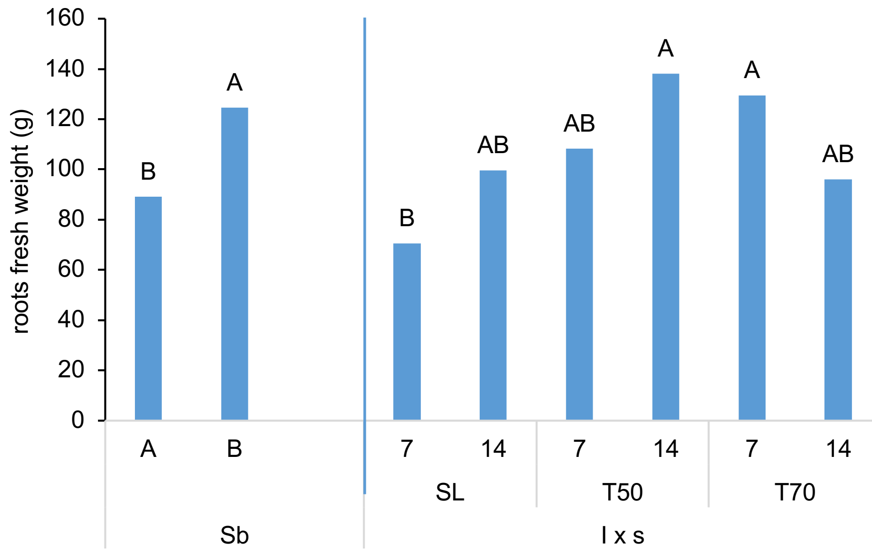

670 **Figure 9**. *Aloe arborescens*. Fresh weight of roots (g) averaged by substrate (Sb: A or B), and means of the I x s (Illumination
671 x stress) interaction. Within each group, different letters indicate a significant difference (P ≤ 0.05; Tukey's test) among
672 the treatment means.
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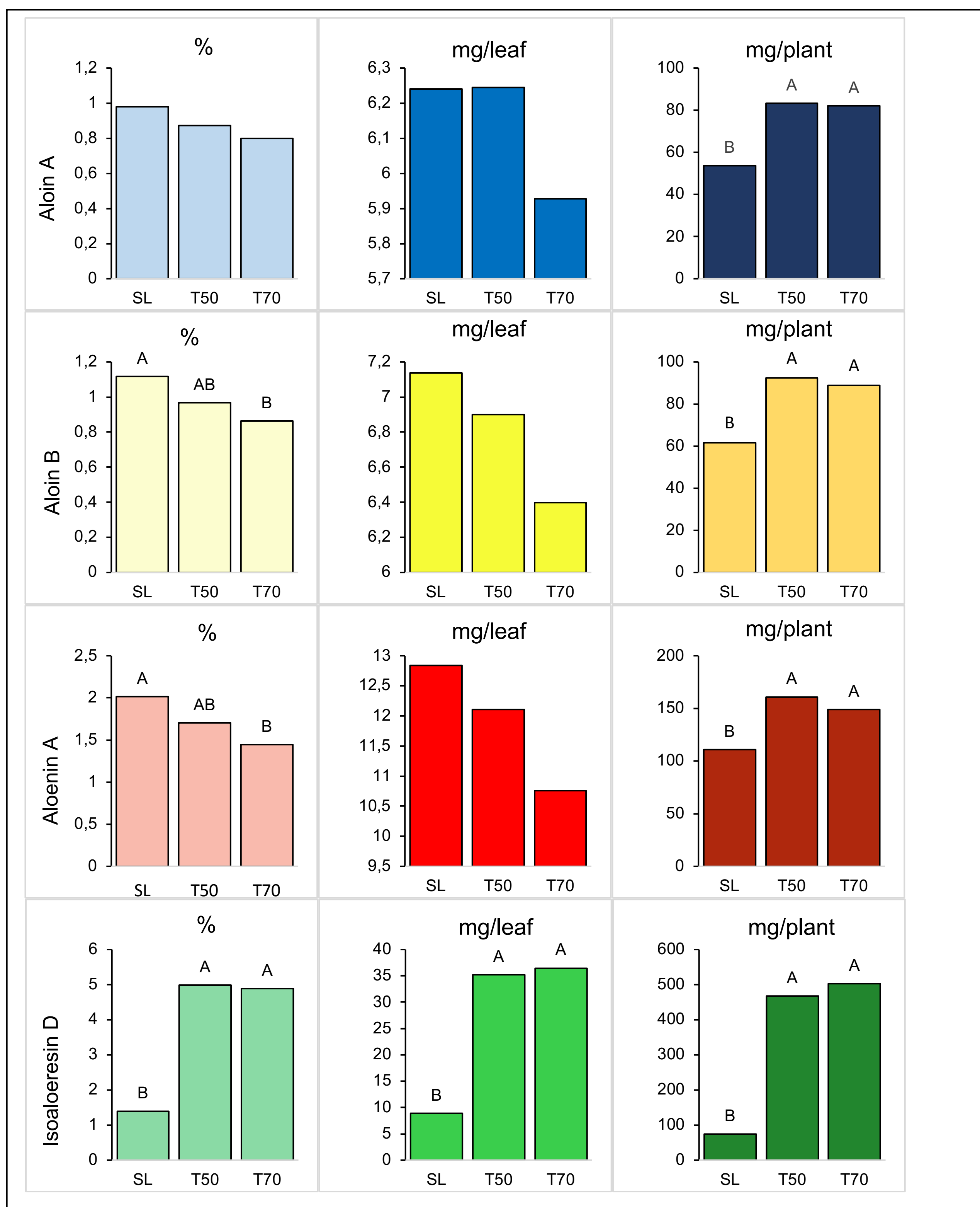

678 **Figure 10**. *Aloe arborescens*. Content in active metabolites according to illumination level. From top to bottom: Aloin A, Aloin B,
679 Aloenin A and Isoaloeresin D. From left to right: measured content in % d.m. and estimated content in mg leaf^{-1} and mg plant^{-1}.
680 Within each graph, different letters indicate a significant difference (P ≤ 0.05; Tukey's test) among the means.