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Secondary metabolites of some varieties of *Caulerpa species*

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Abstract. Several species of *Caulerpa* have been investigated and bioactive principles, such as caulerpin and caulerpicine have been isolated from a number of them. Caulerpin produces mild anesthetic action, difficulty in breathing, sedation, and loss of balance. The toxic syndrome has been reported to be somewhat similar to that produced by ciguatera fish poisoning. The neurotrophic activity of caulerpicine is thought to be of clinical value. Tropical green algae and a few of their temperate relatives have yielded a number of bioactive metabolites and some of these are believed to be used by the algae as a chemical defense against herbivorous animals. This research was aimed to determine the secondary metabolites compound of some varieties of *Caulerpa species*. Chromatography and Spectrophotometric methods were used to conduct this research. The result shows that Caulerpin and caulerpicin presence on five species of *Caulerpa* species *Caulerpa racemosa* Siquijor, *Caulerpa lentillifera* wild and farm from Mactan, *Caulerpa clavifera* Mactan and *Caulerpa lentillifera* cultured in the common garden. Higher absorbance of both compounds caulerpin and caulerpicin found in wild *Caulerpa lentillifera* from Mactan while the lowest absorbance of caulerpin found in *Caulerpa racemosa* collected from Siquijor, whereas the lowest absorbance of caulerpicin found in the cultured *Caulerpa lentillifera* in the common garden.

1. Introduction

The most popular edible algae in the Philippines are considered toxic during the rainy season. This toxicity has been attributed to the presence of caulerpicine which has been found to have neurotrophic effects. Its toxicity, presumed to be a chemical defense of the alga.

Several species of *Caulerpa* have been investigated and bioactive principles, such as caulerpin and caulerpicine have been isolated from a number of them. Caulerpin produces mild anaesthetic action, difficulty in breathing, sedation and loss of balance. The toxic syndrome has been reported to be somewhat similar to that produced by ciguatera fish poisoning. The neurotrophic activity of caulerpicine is thought to be of clinical value. Tropical green algae and a few of their temperate relatives have yielded a number of bioactive metabolites and some of these are believed to be used by the algae as a chemical defense against herbivorous animals [1]. The concentration of caulerpenyne varies with water depth and temperature but is always higher in the invasive strain [2]. Water

temperatures above 19 ^{o}C (spring to autumn) and depths of around 5 m are the optimum conditions for caulerpenyne production [3].

Secondary metabolites produced by *Caulerpa* species, being the toxic terpenoid caulerpenyne Ranielo et al. [4], the most studied, have been investigated to evaluate their ecological role in chemical defense against herbivores while no studies were performed to investigate their possible role as allelochemicals in interspecific competition among marine macrophytes. Dumay et al. [5] provided interesting data about changes in caulerpenyne content in the two invasive species of *Caulerpa, C. taxifolia* and *C. racemosa* var. *cylindracea*. These two algae showed a decrease in caulerpenyne content in the presence of *P. oceanica*, with a concomitant increase in frond length. Further, when growing under the seagrass canopy, the *Caulerpa* species may allocate more energy into frond development to the detriment of other functions such as the production of secondary metabolites.

The marine alga *Caulerpa lamourouxii* is widely distributed in the Phillippines. The upper branches are eaten as a 'salad', despite their peppery and astringent taste. However, the alga is found toxic to some individuals. Chemical investigation of the alga had furnished caulerpicine, caulerpin, cholesterol, taraxerol, β-sitosterol and palmitic acid. Caulerpin had also been isolated from *Caulerpa sertularioides, C. racemosa* var. *clavifera* and caulerpicine *from C. racemosa* [1].

Among the many secondary metabolites produced by algae belonging to the genus *Caulerpa*, caulerpenyne (CYN) is the most abundant cytotoxic sesquiterpenoid to be released. In *Caulerpa taxifolia* (Vahl) C. Agardh, this molecule makes up 0.2% (in spring) to 13% (in summer) of the alga's frond wet weight [5], values that are much higher than those observed in other *Caulerpa* species [6]. In *C. taxifolia* and *C. racemosa* (Forssk°al) J. Agardh, CYN, thought to be the most active substance, is involved either in the chemical defense of the plant against herbivore pressure or within the framework of interspecific competition (antifeedant and antifouling effects) [5,7]. A variety of toxic effects due to CYN has been demonstrated in several organisms at different stages of their growth. The most harmful effect is on sea urchin eggs, which are killed by CYN [7].

The selective force of specialist herbivores is considered an important driver in the evolution of quantitative and qualitative variation in plant chemical defenses [8,9]. For this reason, linking variation in seaweed chemical defenses to the diet specificity of marine herbivores [9,10] is an important precursor to addressing theory derived from specialist associations in terrestrial systems, in particular, Baumgartner et al., [9]. Because most marine herbivores (fishes, sea urchins, and gastropods) are generalists [11], examples of chemically mediated, marine specialist interactions come from a broader phylogenetic pool of species than the traditional plant-herbivore model [9].

However, 2 groups of herbivores (amphipods and sacoglossan opisthobranch mollusks) do contain consumers with consistently narrow host ranges [10,11]. Of these, the sacoglossans are almost exclusively associated with chemically rich seaweeds (siphonous green algae) [12], which provides an opportunity to contrast the efficacy of chemical defenses in closely related seaweeds against multiple specialized herbivores. Feeding preferences could have selected for high levels (an upper threshold) in *Caulerpa* chemical defense, in an attempt to radiate and escape as suggested for terrestrial plants in response to specialization by herbivores [9]. Similarly, any preference for seaweed with intermediate concentrations could manifest as an indirect defense for low concentration *Caulerpa* species (e.g. *C. racemosa* var. *laetevirens*), where such species are common but are avoided by sacoglossans with broader diet niches (e.g. *Elysia* and *Lobiger*) [9].

Raniello et al. [4] demonstrated that the presence of the invasive seaweed in the meadows stimulated the sexual reproduction of seagrasses, reported by many authors to be more frequent under stressful conditions [4,13]. However, the determinants of the observed structural and physiological changes induced in the benthic community have not been fully investigated, although Ceccherelli et al. [14] have speculated on the possible occurrence of an allelopathic interaction by which the release of a phytotoxic compound would affect and damage the competitor physiology. The aims of this study are to determine the secondary metabolites compound of some varieties of *Caulerpa* species using chromatography and spectrophotometry methods.

2. Materials and Methods

2.1. Sample collection

Five species of *Caulerpa* were collected from different sites, *Caulerpa lentillifera wild, Caulerpa lentillifera farm, and Caulerpa clavifera* were collected from Mactan island, *Caulerpa racemosa* were collected from Siquijor, and *Caulerpa lentillifera* was collected from the experiment tanks of Institute of Environment and Marine Sciences (IEMS) Silliman Laboratory (common garden cultured *Caulerpa lentillifera*).

2.2. Sample preparation

Fresh and healthy samples of each species were collected and cleaned from epiphytes, muddy sands and other dirt. Samples were air-dried for 5 to 7 days until they become well dried and was ground to the powder form. Modified method by Doty and Santos [15].

2.3. Sample extraction

The grounded sample was weighed 100 grams and refluxed in 1000ml of 95% ethanol for 3 days at 70^{o}C. A minimum of 10 milliliter ethanol per dry gram of sample was used for each refluxing. After the three days refluxing the ethanol was collected by filtering to separate the extract from the debris.

2.4. Partitioning

Ethanol extracts were concentrated on a Brinkman flash evaporator. Equal volumes of diethyl ether were added to the ethanol and samples were set aside for 24 hours. The samples were filtered and evaporated to dryness.

Equal volumes of ether and water were added to make it 100ml and two layers were separated using a separatory funnel. The bottom part contains water that was discharged and the ether upper layer containing compounds were collected and concentrated to a small volume.

2.5. Chromatography

The separated and purified diethyl ether extract was adsorbed onto 100g neutral alumina in a column (16cm x 4.5cm) and eluted with diethyl ether.

Fractions were concentrated and spotted on silica gel coated thin layer chromatographic plates and were developed using the solvent system made up of hexane: diethyl ether: acetic acid in 30:70:1 ratio.

2.6. Retention factor (Rf) analysis

The spots developed on the TLC plate were marked and measurement of Rf value was done to determine the compounds. The UV light was used to visualized and mark the Rf value of the colorless compound caulerpicin.

2.7. Spectrometric analysis

Spots on the TLC plates scraped and were dissolved in diethyl ether and ethanol. The absorbance spectra for each spot was determined using the wavelength scan mode of the Perkin Elmer Lambda 100 Spectrophotometer. For caulerpin the absorbance spectra were generated using the wavelengths from 200 to 600 nm, while for caulerpicin the wavelength used was from 200 to 350 nm.

3. Results

3.1. Column Chromatography

Five bands were obtained from the column chromatography perform on all species *Caulerpa racemosa* Siquijor, *C. lentillifera* wild and farm from Mactan, *Caulerpa clavifera* Mactan and *Caulerpa lentillifera* cultured in the tank IEMS.

Tabel 1. Number of fraction yielded on column chromatography

No	Species	Number of Fractions
1	*Caulerpa lentillifera* Farmed – Mactan	5
2	*Caulerpa lentillifera* Wild – Mactan	5
3	*Caulerpa lentillifera* Tank	5
4	*Caulerpa clavifera* – Mactan	5
5	*Caulerpa racemosa* – Siquijor	5

The column chromatography test results shown in Table 1, summarizing the five *Caulerpa* species from five different locations, each has five fractions. Which is means that there are five possibilities compounds content in *Caulerpa* species from five species.

Caulerpin found on the first three fractions of column chromatography of each sample, while caulerpicin found at the last fraction column chromatography. The first fraction was dark green color, second fraction light green, third fraction light green (brownness), forth fractions light green to yellow color and the last fraction dark green. All fractions were subjected to thin-layer chromatography (TLC).

3.2. Thin Layer Chromatography

Fractions collected from the column chromatography were subjected to the Thin Layer Chromatography, this technique for chemical separation of mixtures compounds or substances content on the fractions collected from column chromatography. Fourteen to sixteen bonds were generated from the thin layer chromatography for all five *Caulerpa* species extracted,

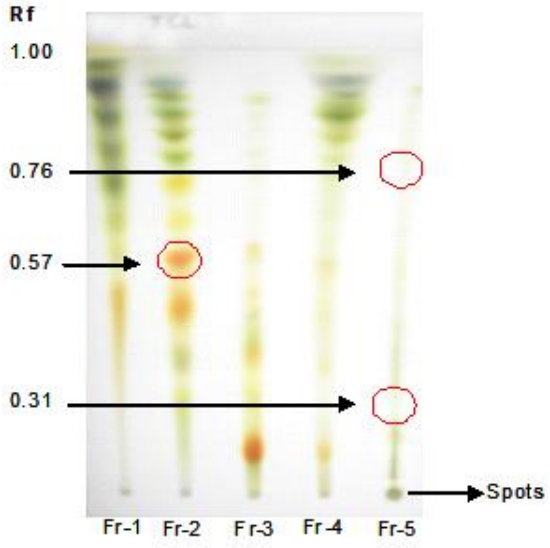


Figure 1. Thin Layer Chromatography result (TLC) of *Caulerpa* species.

The result of Thin Layer Chromatography (TLC) on the second fraction showed the bright orange color in the red circle with Rf value 0.57 which is the characteristic color of caulerpin, while the colorless spot of caulerpicin was obtained from the fifth fraction had two spots with the first spot with Rf value of 0.31 and the second is 0.76. While the yellow color spot with an Rf of 1.00 was identified as α-carotene pigments, chlorophyll pigments as indicated by the green color spots. Some other spots present in the TLC plates were not been identified (Figure 1).

Table 2. Retention factor (Rf) Value of TLC product of *Caulerpa* species

No	Species	Caulerpin		Caulerpicin	
		Presence (+), absence (–)	Rf	Presence (+), absence (–)	Rf
1	*C. lentillifera* Farmed – Mactan	+	0.575	+	0.31
2	*C. lentillifera* Wild – Mactan	+	0.575	+	0.31
3	*C. lentillifera* –Tank	+	0.575	+	0.31
4	*C. clavifera* – Mactan	+	0.566	+	0.31
5	*C. racemosa* – Siquijor	+	0.575	+	0.31

Table 2 shows that caulerpin presents in the 4 *Caulerpa* species extracted with the Retention factor (*Rf*) value of 0.575, except *Caulerpa clavifera*; caulerpin presents at *Rf* 0.566; while caulerpicin presents in all extracted *Caulerpa* species with *Rf* value of 0.31.

3.3. Spectrophotometric analysis

The spectrophotometer on this study was used to measure the intensity of wavelengths in a spectrum of light compared with the intensity of light from a standard source of five *Caulerpa* species.

Table 3. Wavelength and absorbance value of Caulerpin

No	Species	Caulerpin					
		Wavelength (nm)			Absorbance		
1	*Caulerpa lentillifera* Farmed – Mactan	445	427	408	0.559	0.652	0.604
2	*Caulerpa lentillifera* Wild – Mactan	445	428	408	0.707	0.813	0.735
3	*Caulerpa lentillifera* –Tank	445	426	405	0.411	0.497	0.424
4	*Caulerpa clavifera* – Mactan	445	431	408	0.399	0.411	0.386
5	*Caulerpa racemosa* – Siquijor	445	427	408	0.217	0.295	0.304

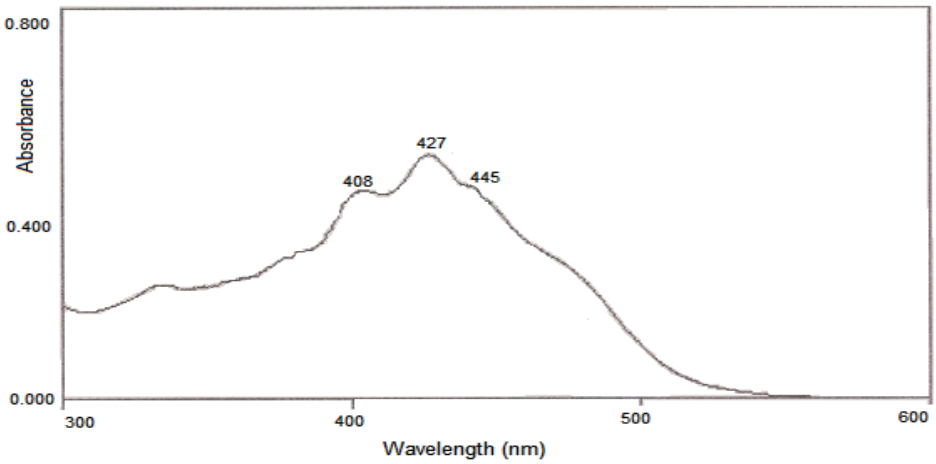
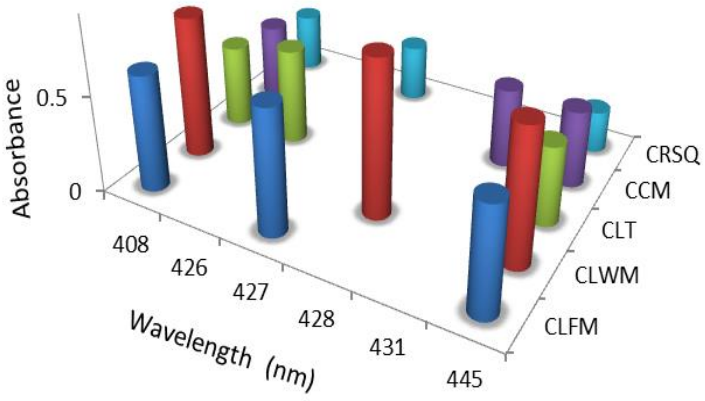


Figure 2. Spectrogram of caulerpin.

Figure 2 showed the spectrogram of caulerpin, which composed of 3 peaks (408 – 427 – 445), while Figure 4 and Table 3 show the wavelength and the corresponding absorbance of caulerpin extracted from five species extracted. The result of spectrophotometer showed that caulerpin absorbed light at wavelengths 408nm to 445nm.



CRSQ: *Caulerpa racemosa*-Siquijor
CCM: *Caulerpa clavifera*-Mactan
CRSQ: *Caulerpa lentillifera*-tank
CLWM: *Caulerpa lentillifera* wild-Mactan
CLFM: *Caulerpa lentillifera* farm-Mactan

Figure 3. Amount of light absorbed by caulerpin from five *Caulerpa* species at various wavelengths.

Table 3 and Figure 3 showed that *Caulerpa lentillifera* wild taken from Mactan had the highest absorbance of caulerpin, which is (0.707 – 0.813 – 0.735) at the wavelength (445 – 428 – 408) nm, followed by *Caulerpa lentillifera* farm from Mactan with absorbance of caulerpin (0.559 – 0.652 – 0.604) at the wavelength (445 – 427 – 408) nm, while the lowest absorbance found on *Caulerpa racemosa* from Siquijor (0.217 – 0.295 – 0.304) at the wavelength of (445 – 427 – 408) nm.

Table 4. Wavelength and absorbance value of caulerpicin

No	Species	Caulerpicin	
		Wavelength (nm)	Absorbance
1	*Caulerpa lentillifera* Farmed – Mactan	208	0.313
2	*Caulerpa lentillifera* Wild – Mactan	214	0.324
3	*Caulerpa lentillifera* –Tank	216	0.290
4	*Caulerpa clavifera* – Mactan	217	0.304
5	*Caulerpa racemosa* – Siquijor	218	0.295

Table 4 shows the wavelength and the absorbance value of caulerpicin obtained from five species extracted. The result of spectrophotometer showed that Caulerpicin absorbed light at wavelengths 208nm to 218nm.

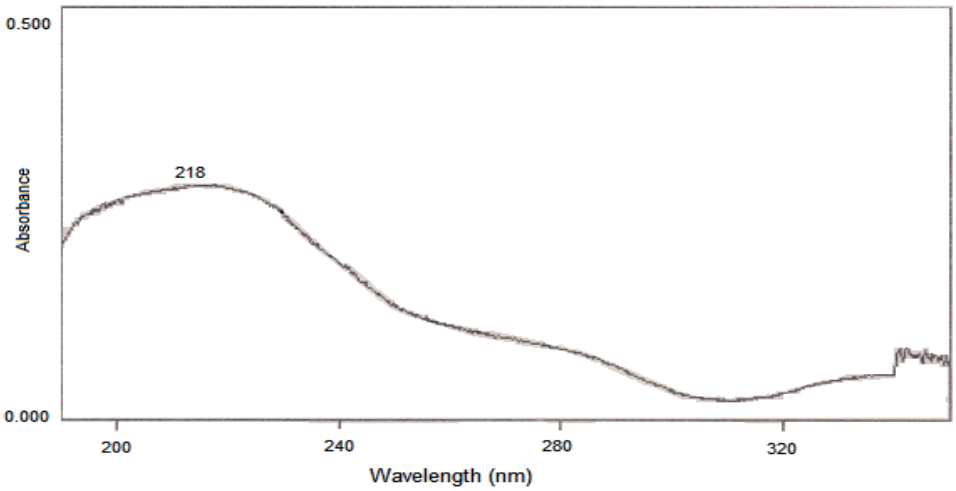
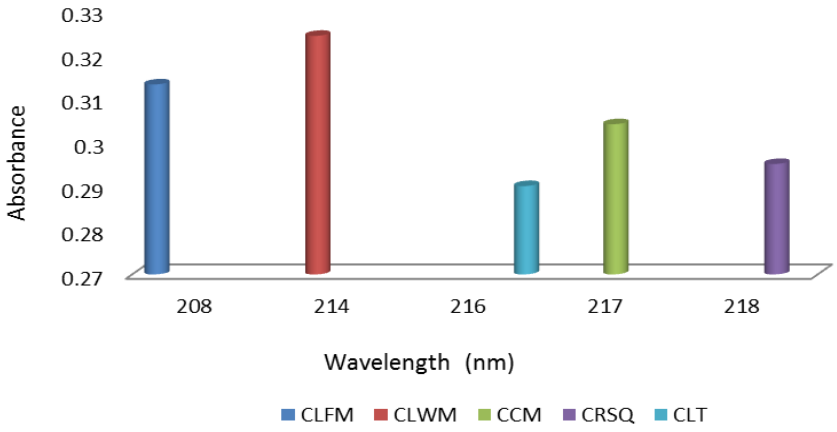


Figure 4. Spectrogram of Caulerpicin.

Figure 4 showed the spectrogram of caulerpicin which appeared one peak located between 200 to 230 nanometer, while Figure 5 showed the amount of light absorbed by caulerpicin from five *Caulerpa*.



CRSQ: *Caulerpa racemosa*-Siquijor
CCM: *Caulerpa clavifera*-Mactan
CRSQ: *Caulerpa lentillifera*-tank
CLWM: *Caulerpa lentillifera* wild-Mactan
CLFM: *Caulerpa lentillifera* farm-Mactan

Figure 5. Amount of light absorbed by caulerpicin from five *Caulerpa* species at various wavelengths.

The highest absorbance of caulerpicin found in *Caulerpa lentillifera* wild from Mactan Island with value of 0.324 located in 214 nm of wavelength. The second highest found in *Caulerpa lentillifera* farm from Mactan with absorbance value of 0.313 at the wavelength 208 nm, while the lowest absorbance of caulerpicin found on *Caulerpa lentillifera* cultured on tank with the absorbance value of 0.290 at the wavelength of 216nm.

4. Discussion

Based on the result caulerpin were present in the first to third fraction, while caulerpicin found in the fifth or last fraction, in the study of eight species of green algae of *Caulerpa*, Vest [16] found five to eight bands in the column chromatography, caulerpin were eluted in the first three bands while caulerpicin appeared in the last band or the column wash.

The bright orange color on the Thin Layer Chromatography (TLC) at the Rf of 0.57 was characteristic color of caulerpin, while the colorless spot of caulerpicin at the Rf 0.31 was obtained from the fifth fraction, this result similar with the study of Vest [16], caulerpin appeared as a single bright orange band with an Rf value of 0.58, while caulerpicin appeared as colorless compound with an Rf value of 0.32. Bhakuni and Rawat [1] had been isolated Caulerpin from *Caulerpa sertularioides, C. racemosa* var. *clavifera* and caulerpicin from *C. racemosa*.

According to Valerie [17] Caulerpin is often a minor metabolite in *Caulerpa*. This bright-orange, pigmented compound, probably derived from indole biosynthesis, has been found in over 50% of the *Caulerpa* species [18,19].

Other color spots were also obtained from TLC. A yellow color spot with an Rf of 1.00 was identified as α-carotene pigments, chlorophyll pigments as indicated by the green color spots. Some other spots present in the TLC plates were not been identified. Dawes [20] and Goodwin [21] stated that carotene pigments located in Rf of 1.0 with the range of yellow to red color.

Since in this study, the pure caulerpin and caulerpicin as standard compounds were absent, the compounds were investigated only by comparing the relative abundance by measuring the absorbance in spectrophotometer. *Caulerpa lentillifera* wild taken from Mactan had the highest absorbance of caulerpin, followed by *Caulerpa lentillifera* farm from Mactan while the lowest absorbance found on *Caulerpa racemosa* from Siquijor. Study of Vest et al. [16] in eight species of green algae *Caulerpa*, caulerpin was present in *C. cupressoiedes, C. paspaloides, C. prolifera and C. sertularioides* in amounts varying from less than 1 mg to 5.47 mg per dry gram of algae.

Same as caulerpin, highest absorbance of caulerpicin found in *Caulerpa lentillifera* wild from Mactan, while the lowest absorbance of caulerpicin found on *Caulerpa lentillifera* cultured on tank. Study of Vest [18], caulerpicin was found in *C. ashmeadii, C. paspaloides, C. sertularioides,* and *C racemosa var. uvifera* in lesser concentrations ranging from 0.03 to 0.09 mg per dry gram of algae.

Wild *Caulerpa lentillifera* had the highest caulerpin and caulerpicin, this may be related to environmental factors which *Caulerpa* will produce more caulerpin and caulerpicin as prevention from the predators compare to the farmed and the tank which less in predators. According to Ruensink [22] *Caulerpa* produces secondary metabolites such as caulerpicin that repel predators.

5. Conclusion

Caulerpin and caulerpicin presence on five species of *Caulerpa* species *Caulerpa racemosa* Siquijor, *Caulerpa lentillifera* wild and farm from Mactan, *Caulerpa clavifera* Mactan and *Caulerpa lentillifera* cultured in the common garden.

Higher absorbance of both compounds caulerpin and caulerpicin found in wild *Caulerpa lentillifera* from Mactan while the lowest absorbance of caulerpin found in *Caulerpa racemosa* collected from Siquijor, whereas the lowest absorbance of caulerpicin found in the cultured *Caulerpa lentillifera* in the common garden.

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