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Wound plug chemistry and morphology of two species of *Caulerpa* – a comparative Raman microscopy study

Abstract: *Caulerpa* spp. form a polymer wound plug that seals their giant cells after mechanical injury, in order to prevent fatal loss of cell material. Initial mass spectrometry and Raman spectroscopy investigations revealed the involvement of the secondary metabolite caulerpenyne in wound sealing polymer formation. In this work, we introduce a comparative Raman spectroscopic study of the wound plug formation in the invasive *Caulerpa taxifolia* (Valh) Agardh, 1817 and the non-invasive *Caulerpa prolifera* J.V. Lamour. In both species, the enzymatic transformation of the main secondary metabolite caulerpenyne plays a key role in wound plug formation. An accumulation of caulerpenyne is observed at the inner border of the wound plugs. Furthermore, caulerpenyne and products resulting from its enzymatic transformation and co-polymerization with proteins are found within the wound plug. However, there are significant differences in the chemistry of the wound plugs between the species. The Raman spectra reveal a zonation of the wound plug of *C. taxifolia* into four chemically distinguishable regions, while that of *C. prolifera* consists of only three regions with specific chemical composition. These results explain differences in the morphology of the wound plug in both species.

Keywords: asexual reproduction; *Caulerpa prolifera*; *Caulerpa taxifolia*; wound plug formation; wound reaction.

Introduction

The two species, *Caulerpa taxifolia* and *Caulerpa prolifera*, grow in the Mediterranean Sea, but while *C. prolifera* belongs to the native vegetation, *C. taxifolia* is an invasive species (Williams 2007). There are several possible reasons for the success of *C. taxifolia*, including the exceptional temperature tolerance of this initially tropical species, its rapid growth, and its chemical defense mechanisms (Box et al. 2010, Montefalcone et al. 2010, Infantes et al. 2011, Varela-Álvarez et al. 2012). Additionally, the effective asexual reproduction based on mechanical fragmentation of *C. taxifolia* and subsequent distribution of the fragments contributes to its spread (West et al. 2007). This strategy of asexual reproduction is particularly remarkable considering that *Caulerpa* species can reach meters in length with a unicellular siphonous organization.

A wound plug is formed within the first seconds after an injury to prevent the loss of cell content (Dreher et al. 1982, Menzel 1988, Welling et al. 2009). Resulting sealed fragments of the multinucleate alga can then regrow as independent clones (Jousson et al. 2000, Meinesz et al. 2001). The morphological development of the wound plug of Caulerpales has been well described and the fundamental biochemical transformations leading to its formation have also been characterized (Menzel 1988, Welling et al. 2009). Upon wounding of *C. taxifolia* and *C. prolifera*, the main secondary metabolite caulerpenyne is enzymatically transformed to oxytoxin 2, which crosslinks algal proteins (Figure 1) (Jung and Pohnert 2001, Adolph et al. 2005, Weissflog et al. 2008). Cross-linking results in polymer formation thus contributing to the sealing of the wounds. While species possess comparable amounts of caulerpenyne, the rate of transformation differs substantially (Jung et al. 2002). In a model experiment by Jung et al. (2002), the concentration of caulerpenyne in *C. prolifera* decreased to 11% of its initial level within one minute after wounding. In contrast, the concentration of caulerpenyne decreased significantly more slowly in *C. taxifolia*. While the overall chemical mechanism of caulerpenyne transformation has been found to be similar in both algae, the metabolite is

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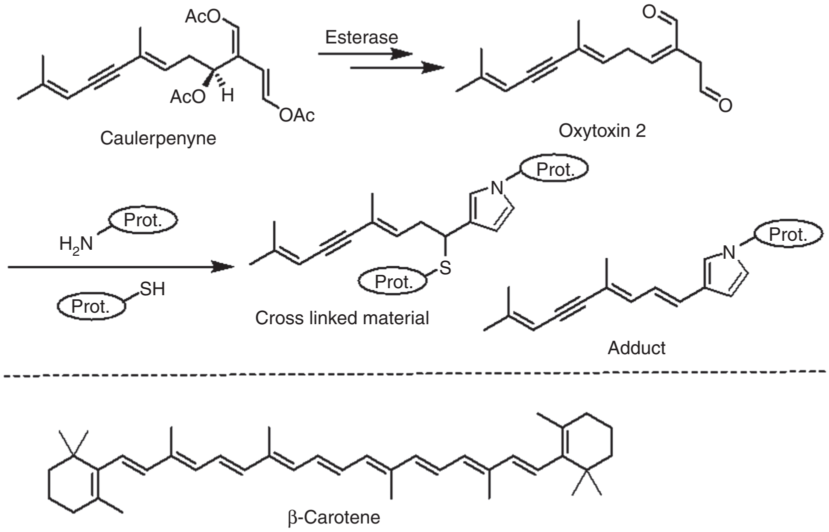

Figure 1 Top: Chemical structure of caulerpenyne and the chemical processes occurring during wound plug formation (Prot.=protein) (after Adolph et al. 2005). Below: Chemical structure of β-carotene.

still present at 50% of the initial concentration one minute after wounding in *C. taxifolia*. Moreover, a comparison of the wound plugs of the two species reveals morphological differences (Jung et al. 2002). A possible chemical difference between the wound plugs of both *Caulerpa* species is difficult to prove by common analytical methods, as the material is insoluble and the constituents are of high molecular weight (Adolph et al. 2005). Therefore, we reverted to Raman spectroscopy to obtain chemical information about different parts of the wound plug without destruction of the sample (Weissflog et al. 2013). This spectroscopic method requires minimum sample preparation, and no disruption of the wound plug occurs due to stress during measurements (Walter et al. 2010, Grosser et al. 2012).

The comparative study presented here follows up a first report on the wound plug formation of *C. taxifolia* (Weissflog et al. 2013). In this previous study, it has been reported that the region around the wound site of *C. taxifolia* consists of four chemically distinct zones. Here, we reveal distinct differences between the species in the chemical composition of the wound plug region with respect to the protein cross-linking derivatives of caulerpenyne. The results can provide a chemical rationale for the observed morphological differences in the wound plug regions of both algae.

Materials and methods

The wound plugs of 26 individuals of *Caulerpa prolifera* and 13 individuals of *Caulerpa taxifolia* were analyzed in artificial seawater (Instant Ocean, Aquarium Systems, Blacksburg, VA, USA), using the motorized horizontal sample video stage-configuration of a FT-Raman device (Bruker, Bremen, Germany). Spectra were recorded at an excitation wavelength of 1064 nm with a laser power at the sample of 800 mW. Line mapping was performed with a point-to-point distance of 100 μm, which was the size of the laser spot. A spectral resolution of 2 μm^{-1} was chosen, and each spectrum was averaged in 30 scans. All spectra were analyzed using R (R Development Core Team 2010). Baseline correction was carried out using the method SpectrumBackground of the package Peaks (Morhac 2008), working with a 4th order clipping filter. Subsequently, all spectra were normalized to the area of the sum of the CH_2-vibration bands between 2836 and 3033 cm^{-1} and then sorted by their different spectral components, e.g., the characteristic Raman bands for the wound plug. The spectra were averaged after sorting into different zones. For the plots showing the Raman intensities of characteristic bands vs. the distance from the cut, the integrated intensities of those bands were calculated for each spectrum and related to the distance of each measurement point from the cut.

Sample preparation

For FT-Raman measurements, individuals of *C. taxifolia* and *C. prolifera* were cut between the stolon and the broad leaf-like portion of the thallus (assimilator) with a scalpel. To support the development of the wound plug, the cut was performed under water (Hawthorne et al. 1981). After

2–5 min, when the wound plug was fully developed, the individuals were mounted on a silica glass slide and wet with seawater. Using hot glue, a glass frame was constructed from two capillaries. The samples were mechanically fixed onto the cover slide using this frame. Background measurements of the experimental setup without a sample revealed that neither the glue nor the holding capillaries contributed to the analyzed Raman signals.

Algae were controlled for desiccation and for signs of cytoplasmic streaming after the measurements. Only measurements where no damage of the experimental organisms was observed were considered for further analysis.

Cultivation

C. taxifolia and *C. prolifera* were purchased from different aquarium shops and kept in aerated aquaria. The aquaria were filled with 7–20 l of artificial seawater (Instant Ocean, Aquarium Systems, PSU 24), and the water temperature was kept constant at 22°C. The photo regime was operated with constant illumination (40 μmol m^{-2} s^{-1}) for 14 h followed by a 10-h dark period.

Results and discussion

The chemical composition of wound plugs in siphonous macroalgae differs substantially between different genera (Menzel 1988, Adolph et al. 2005, Welling et al. 2011). In this comparative study of wound plug formation, more subtle differences in wound plug chemistry were demonstrated at species level within the genus Caulerpa. This comparative investigation was performed with *Caulerpa taxifolia* and *Caulerpa prolifera*, which were cut under water between the stolon and the assimilator with a razor blade to initiate the wounding in a reproducible manner. After 2–5 min, when full development of the wound plug was observed, the individuals were mounted on a silica glass slide and wet with sea water. Raman spectra were recorded with a step size of 100 μm from the external wound plug to 2–3 mm within the tissue. The resultant spectra were classified in terms of significant changes and then grouped into distinguishable zones at different distances from the cutting site. Within similar regions, spectra were averaged and areas with distinct chemical compositions were classified. Additionally, intensity profiles of prominent Raman bands related to the distance from the cut were determined for individual wound plugs.

Upon visual inspection, the wound plug of *C. taxifolia* (Figure 2(B)) can be considered to be more efficient than that of *C. prolifera* (Figure 2(D)). For *C. prolifera*, more cell content is lost due to the formation of a larger external wound plug, which is a process that causes more extensive damage to the stolon.

The averaged spectra of wound plugs from both species showed significant contributions of β-carotene at 1156 and at 1527 cm^{-1} (Figure 3). As an accessory pigment for photosynthesis, β-carotene is associated with the plastids. Given that plastids are found in the cytoplasm, local

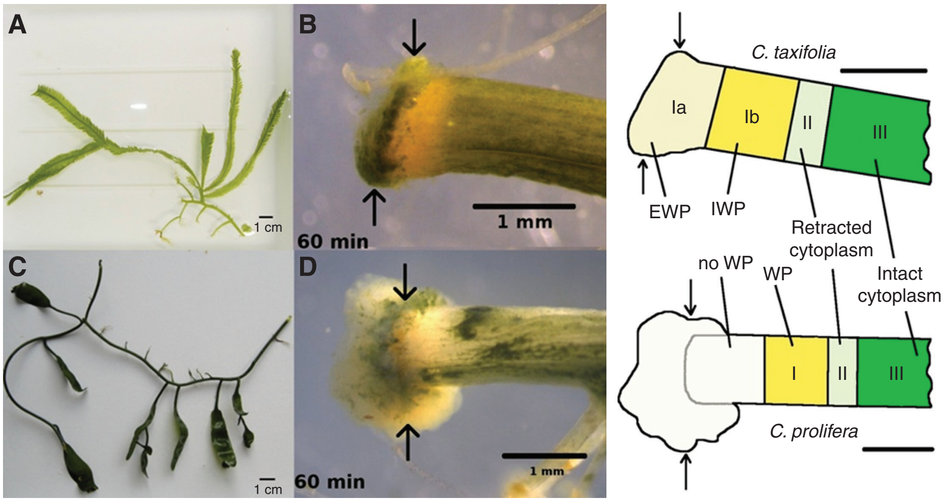

Figure 2 (A) Morphology of *Caulerpa taxifolia*, (B) wound plug of *C. taxifolia* 60 min after wounding, (C) morphology of *Caulerpa prolifera*, (D) wound plug of *C. prolifera* 60 min after wounding. (E) Schematic diagram of the wounded area of *C. taxifolia* and *C. prolifera* based on Raman spectroscopic results. Scale bars=1 mm. Arrows in (B), (D), and (E) indicate positions of the cut; WP, wound plug; EWP, external wound plug; IWP, internal wound plug.

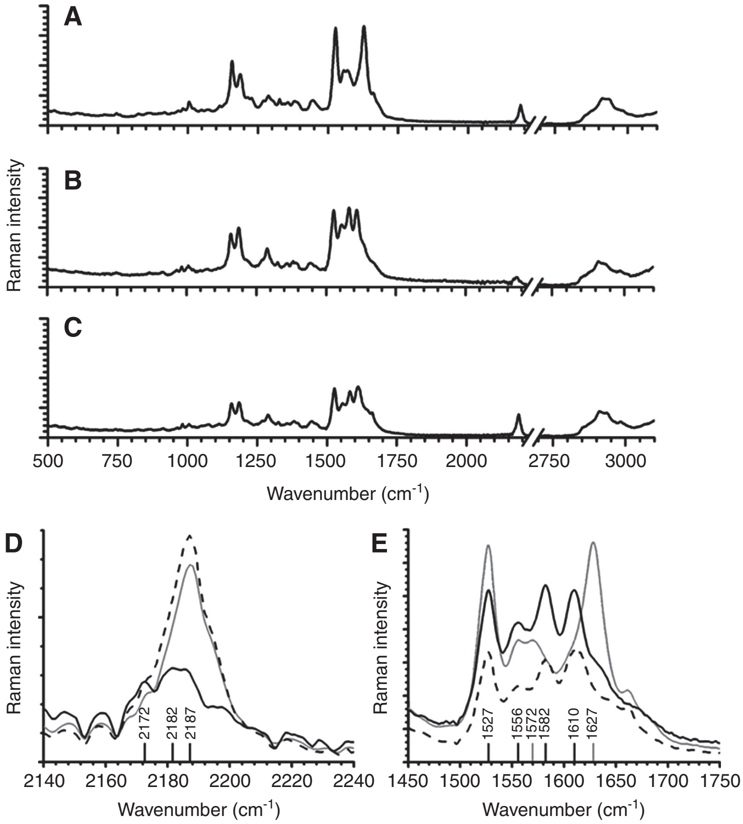

Figure 3 The averaged spectrum of the wound plug of *Caulerpa prolifera* (A), and the external (B) and internal (C) wound plugs of *C. taxifolia*. (D) Superimposed spectra for the band around 2180 cm^{-1} of the external (broken line) and the internal wound plug (black line) of *C. taxifolia* and the wound plug of *C. prolifera* (gray line). (E) Characteristic bands found in the wound plug of *C. prolifera* (gray line), the external (broken line) and the internal wound plug (black line) of *C. taxifolia*.

maxima are expected there. The intensities of the bands vary between the single spectra within a wound plug and between individual wound plugs due to randomly captured plastids. To avoid misinterpretation, average spectra were obtained from the 957 single spectra of 26 individuals of *C. prolifera* and 352 single spectra of 13 individuals of *C. taxifolia*. In wound plugs of both species, compared with intact algal tissue, new bands appeared between 1500 and 1700 cm^{-1} (Figure 3(E)), as did a band at 1186 cm^{-1} (Figures 4(A) and 4(B)). In *C. taxifolia*, these signals appeared at 1556, 1582 and 1610 cm^{-1} (Weissflog et al. 2013), while corresponding bands in the wound plug of *C. prolifera* were found at 1556, 1572 and 1627 cm^{-1}. These signals cannot be assigned to specific chemical structures but can be considered as characteristics of the wound plug of the algae. Apparently, the chemical composition is similar overall, but there are subtle differences between the species. Around 2187 cm^{-1}, bands of the carbon triple bond stretching vibration could be observed in both species (Figure 3(D)). These are characteristic of caulerpenyne and caulerpenyne derivatives, which are involved in protein cross-linking (Figure 1) (Weissflog et al. 2013).

Aside from these rather general similarities, the wound plugs of *C. taxifolia* and *C. prolifera* differ distinctly. While two outer chemically defined wound plug regions (Ia and Ib; Figure 2) could be distinguished by the spectral position of the carbon triple bond of caulerpenyne and caulerpenyne-derived compounds in *C. taxifolia*, this is not the case for *C. prolifera*. In the external wound plug of *C. taxifolia* (zone Ia), a shift of the band of the carbon triple bond towards lower wavenumbers compared with caulerpenyne is observed (Figure 3(D)). This band shift is indicative of caulerpenyne-derived cross-linked material formed according to the mechanism depicted in Figure 1. However, the band appeared very weak in contrast to the internal wound plug of *C. taxifolia*. There, the dominant Raman band appeared at the same spectral position as that in caulerpenyne. In contrast, in *C. prolifera*, no region with spectral properties comparable to the external wound plug of *C. taxifolia* can be identified. Only 1.4% of the 507 collected spectra attributed to the wound plug of *C. prolifera* show a shift of the vibration of the carbon triple bond towards lower wavenumbers. The six events where the band is observed could be assigned to regions close to the cutting site within three individuals. In these rare cases, the band of the carbon triple bond is found in half of the spectra at 2172 cm^{-1} or as a shoulder of the band at 2187 cm^{-1} in the second half of the spectra. These data points are surrounded by regions with no observed shift compared to the parent molecule caulerpenyne. Figure 3(D) shows the averaged Raman band of the triple bond in the internal and external wound plug of *C. taxifolia* and the wound plug of *C. prolifera*. Regarding the position and the intensity of the Raman band of the carbon triple bond, the wound plug of *C. prolifera* is comparable to the internal wound plug of *C. taxifolia*.

These differences lead to the question of whether the concentration gradients within the wounded region of *C. prolifera* also differ from those of *C. taxifolia*. Therefore, local concentration gradients were monitored using lateral scans from the external wound plug to the undamaged interior of the algae. Regions of ca. 3 µm were profiled and Raman intensities of prominent bands were recorded. Results are illustrated for one wound plug of each species (Figures 4(A) and 4(B)). The shape of the intensity plots is largely similar, but the locations of maxima and minima within the wound plugs differ between the species. The characteristic band of β-carotene at 1527 cm^{-1} is within the wound plug zones I and Ia of *C. prolifera* and *C. taxifolia*, respectively. This can be assigned to remaining carotene associated with the plastids of captured cytoplasm. In addition, this metabolite is observed in the transition regions to intact tissue (zone III of both algae). The minimum for this band appears around zone 2. Hence, in both species the area of cytoplasm retraction is well reflected by a significantly smaller contribution of β-carotene. The Raman

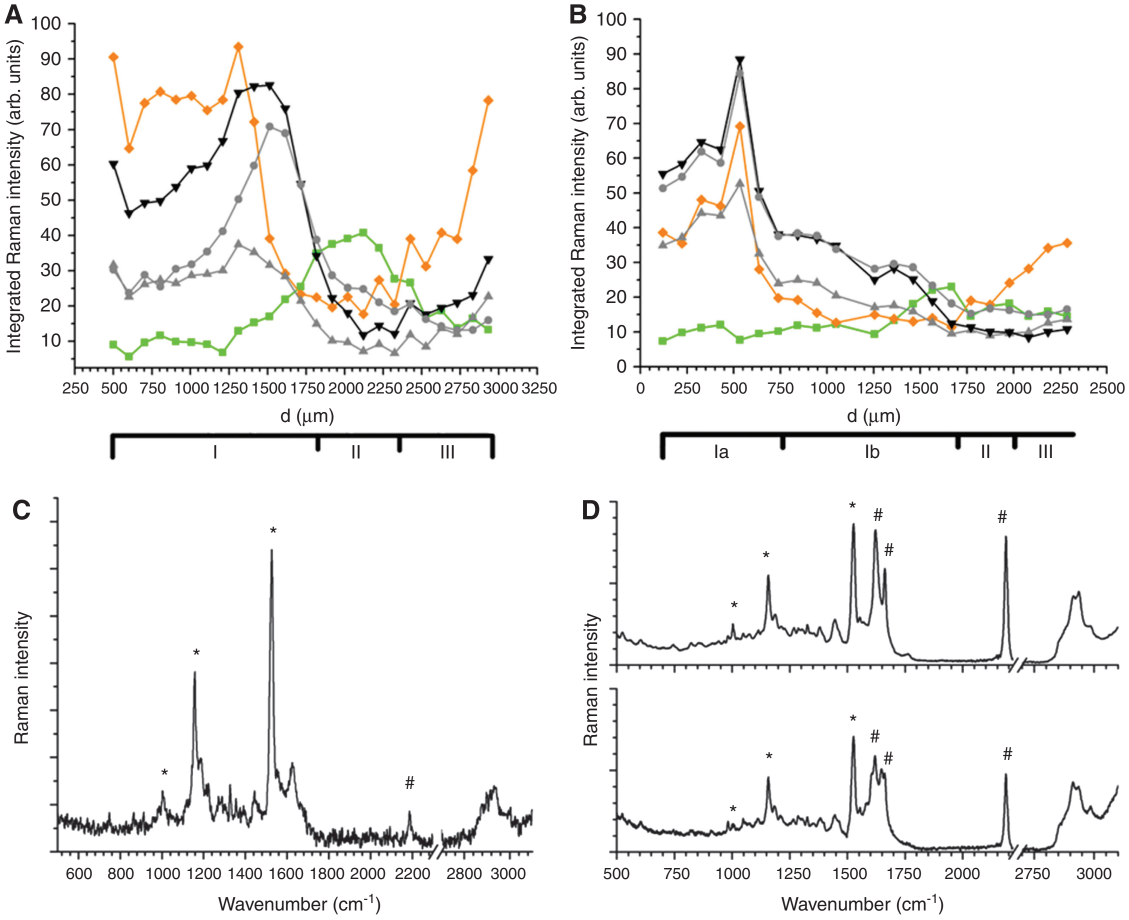

Figure 4 Plots of the Raman band intensities of selected metabolites in relation to the distance from the cut of (A) *C. prolifera* and (B) *C. taxifolia*. The intensity of the Raman bands vs. the distance from the cut are depicted for β-carotene at 1527 cm^{-1} (orange diamonds), the carbon triple bond of caulerpenyne and its derivatives at 2180/2187 cm^{-1} (green squares), as well as the characteristic wound plug bands at 1186 (gray triangles), 1572 (A) or 1582 (B) (gray circles) and 1627 (A) or 1610 (B) cm^{-1} (black triangles). The scales under the graphs indicate the different zones of the wound plugs. (C) Single point spectrum of *C. prolifera* at the site between the cut and the wound plug and (D) the averaged spectra of the area of retracted cytoplasm, top for *C. prolifera* and bottom for *C. taxifolia*. Caulerpenyne and caulerpenyne transformation product derived signals are indicated by #, carotene derived signals by *.

bands that are specifically assigned to the wound plug (1186, 1572, 1582, 1627 and 1610 cm^{-1}) show a comparable gradient to that of β-carotene, except in zone III, where β-carotene reaches levels comparable to the intact tissues, while the wound plug specific metabolites are not observed. Furthermore, in *C. prolifera* only low contributions of the bands assigned to the wound plug were found within the first 900–1500 μm from the cut, when compared with the intensity of β-carotene (Figure 4(C)). A region with comparable spectroscopic signatures could not be identified in *C. taxifolia*. In this study, *C. prolifera* individuals have been observed to have lost cell material for almost half a minute before the wound plug seals the cut. In contrast, the *C. taxifolia* individuals do not show any leakage. In general, the wound plug region extends further into the tissue in *C. prolifera* than in *C. taxifolia*. This is also reflected by the maximum of caulerpenyne, which is caused by recruitment from the tissue located ca. 1.7 mm from the cut in *C. taxifolia* and around 2.1 mm from the cut in *C. prolifera*.

The intensities of the carbon triple bonds of caulerpenyne and its derivatives exhibit a distinct maximum located within zone II of *C. prolifera*, and at the border between the internal wound plug and zone II of *C. taxifolia*. This maximum reflects the accumulation of caulerpenyne at the wounded site as well as the presence of caulerpenyne derivatives within the wound plug. Therefore, in both species, the direct participation of caulerpenyne and its derivatives in the wound reaction is shown *in vivo*.

The FT-Raman spectra of the fully developed wound plugs provide evidence that caulerpenyne, or its derivatives, are present in the wound plug material of both *Caulerpa* spp., and that caulerpenyne is accumulated at the interface between the wound plug region and the internal tissue where the normal cytoplasm is retracted. These findings indicate that caulerpenyne is actively involved in the wound sealing reaction.

The general results concerning caulerpenyne and its derivatives possibly apply to several other caulerpenyne containing *Caulerpa* spp. as well. However, FT-Raman spectra already indicate that there are distinct differences in both the morphologies and chemical compositions of the wound plugs of different *Caulerpa* spp. While *C. taxifolia* forms an external wound plug within the first seconds after wounding and additionally a chemically distinct internal wound plug within the first minute, *C. prolifera* just forms a single chemically homogenous wound plug region. In terms of the spectral contributions of the central metabolite caulerpenyne and its derivatives, the spectroscopic features of the wound plug of *C. prolifera* are comparable with those of the internal wound plug found in *C. taxifolia*. Apparently, *C. taxifolia* relies on an additional mechanism for wound sealing as indicated by the unique properties of the wound plug region Ia (Figure 2). The very weak band of the carbon triple bond in this region suggests that mechanisms other than cross-linking between caulerpenyne derivatives and proteins may be responsible for polymer formation. Dreher et al. (1982) suggested sulphated polysaccharides are involved in a gelling process, which is a mechanism that would be in accordance with our results.

The FT-Raman results presented here are also in agreement with kinetic studies of the wound-activated caulerpenyne transformation (Jung et al. 2002), thus providing a mechanistic insight into the underlying processes that cause observed differences in these kinetics. As such, the significantly slower degradation of caulerpenyne within *C. taxifolia* might be due to the formation of the internal wound plug. In contrast, the faster wound-activated transformation of caulerpenyne in *C. prolifera* might be due to the absence of an external wound plug forming an initial barrier.

This concept can explain the higher efficiency of the wound plug formation, which is apparent already from visual inspection of the wound plugs, that is, the faster formation of the external wound plug in *C. taxifolia* possibly prevents the loss of cell content more efficiently, thus acting as a first protection step of the alga even before the more stable internal wound plug is formed. It can only be speculated if this efficiency might be one reason for the invasive success of *C. taxifolia*. Further studies must focus on the identification of the specific compounds within both the internal and the external wound plugs. These will also shed further light on the mechanism of the formation of the external wound plug.

The results presented here indicate not only fundamental differences in the mechanisms of wound plug formation in different genera of the Siphonales (Menzel 1988, Welling et al. 2009) but also more subtle differences at the species level. These can reflect different adaptation strategies of the algae. The different chemical signatures observed within the wound plug regions point towards specific enzymatic transformations and downstream polymerizations of the common metabolite caulerpenyne in the two species of *Caulerpa* investigated.

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