Induction of wound-periderm-like tissue in Kalanchoe pinnata (Lam.) Pers. (Crassulaceae) leaves as a defence response to high UV-B radiation levels

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BACKGROUND AND AIMS
UV-B radiation can be stressful for plants and cause morphological and biochemical changes. Kalanchoe pinnata is a CAM leaf-succulent species distributed in hot and dry regions, and is rich in flavonoids, which are considered to be protective against UV-B radiation. This study aims to verify if K. pinnata has morphological or anatomical responses as a strategy in response to high UV-B levels.

METHODS
Kalanchoe pinnata plants of the same age were grown under white light (control) or white light plus supplemental UV-B radiation (5 h d⁻¹). The plants were treated with the same photoperiod, photosynthetically active radiation, temperature and daily watering system. Fragments of the middle third of the leaf blade and petiole were dehydrated and then embedded in historesin and sectioned in a rotary microtome. Sections were stained with toluidine blue O and mounted in Entellan®. Microchemical analyses by optical microscopy were performed on fresh material with Sudan III, Sudan IV and phloroglucinol, and analysed using fluorescence microscopy.

RESULTS
Supplemental UV-B radiation caused leaf curling and the formation of brown areas on the leaves. These brown areas developed into a protective tissue on the adaxial side of the leaf, but only in directly exposed regions. Anatomically, this protective tissue was similar to a wound-periderm, with outer layer cell walls impregnated with suberin and lignin.

CONCLUSIONS
This is the first report of wound-periderm formation in leaves in response to UV-B radiation. This protective tissue could be important for the survival of the species in desert regions under high UV-B stress conditions.

Key words: Kalanchoe pinnata, Crassulaceae, leaf anatomy, fluorescence microscopy, radiation responses, UV-B radiation, wound-periderm.

INTRODUCTION
A well-studied environmental stress of plant life is UV-B radiation, mainly due to the increased incidence of this radiation on the surface of the Earth, because of the increasing depletion of the ozone layer (Jansen et al., 1998; Ueda and Nakamura, 2011; Reboredo and Lidon, 2012). UV-B can promote morphological and biochemical changes in plants (Jansen et al., 1998; Jenkins et al., 2014). It can act on DNA, promoting the formation of pyrimidine dimers, increase the generation of ROS (reactive oxygen species) and it can affect plant growth and development (Jansen et al., 1998, 2012). On the other hand, recent discoveries concerning the positive effects of UV-B radiation and its responses, even with low doses, have shifted the perception of this radiation from a stress to a signal (Jansen and Bornman, 2012).

Morphological responses to UV-B can include leaf curling, axillary branching, an increase in the root:stem ratio, inhibition in shoot elongation, and an increase of the leaf area and thickness (Jansen, 2002; Robson et al., 2015). At the cellular level, UV-B can provoke alterations in cell division, elongation and/or differentiation (Jansen, 2002; Robson et al., 2015). Kalanchoe pinnata (Lamark) Persoon (syn.—Bryophyllum pinnatum, B. calycinum) is a member of the Crassulaceae family. The species is commonly distributed in tropical regions (Allorge-Boiteau, 1996; Judd et al., 2009). Crassulacean acid metabolism (CAM) and succulent leaves allow its acclimation to environmental factors such as periodic drought and hot days (Winter, 1985; Judd et al., 2009). In addition, the leaves of this species are rich in flavonoids (Costa et al., 2008), substances that are considered protective against UV-B radiation (Agati et al., 2013), and that also play a role in many of the proven biological activities of this species (Costa et al., 2008). In a previous study, we verified the qualitative and quantitative changes in the phenolic and flavonoid production of K. pinnata leaf extracts, induced by supplemental UV-B radiation (Nascimento et al., 2015). This study aimed to verify whether, besides flavonoid production, this species has morphological or anatomical responses as a strategy to deal with high UV-B levels.
growth chambers for 2 weeks under the same PAR (200–400 μmol m⁻² s⁻¹) of the same age were acclimatized in controlled-environment systems. The PAR was measured with a PAR sensor coupled to an FMS2 Hansatech fluorometer (Hansatech Instruments Ltd., King’s Lynn, UK). Prior to the UV-B treatment, these plants were grown on the campus of the Universidade Federal do Rio de Janeiro, under sunlight, with photosynthetically active radiation (PAR) varying from 400 to 800 μmol m⁻² s⁻¹ and the same watering system. The PAR was measured with a PAR sensor coupled to an FMS2 Hansatech fluorometer (Hansatech Instruments Ltd., King’s Lynn, UK). After 6 months, these plants of the same age were acclimatized in controlled-environment growth chambers for 2 weeks under the same PAR (200–400 μmol m⁻² s⁻¹, depending on plant position), temperature (32 ± 4 °C), manual irrigation (100 mL d⁻¹) and photoperiod (11.5 h of light). Plants were rotated every day, to minimize the effects of positional PAR. These basic growing conditions remained the same throughout the experiment.

Light treatments

After acclimation, the plants were randomly allocated to two different light treatments: white light (W; control) and white light plus supplemental UV-B radiation for 5 h d⁻¹ (from 10:00 h to 15:00 h; W + UVB) (Table 1). Six 59 W daylight fluorescent lamps (Golden, São Paulo, SP, Brazil) were used as a source of white light for both treatments (W and W + UVB); and one tubular 20 W broadband UV-B lamp (290–320 nm; UV-B Medical Phillips TL UV-B G13 T12) was used as a source of supplemental UV-B radiation, only for the W + UVB treatment.

The spectral distribution of the daylight fluorescent lamp radiation was measured with a QMI spectrofluorometer (PTI Inc., Lawrenceville, NY, USA) (Nascimento et al., 2013). The spectral distribution of the UV-B lamp was provided by the manufacturer.

The W + UVB treatment had a mean irradiance of 4–15 W m⁻² (72–200 kJ m⁻² d⁻¹) of UV-B radiation (depending on the plant position), as measured with a VLX-3 W radiometer (with a 312 nm flat sensor, positioned horizontally during the measurements). The corresponding mean biologically effective UV-B irradiance (UVB₉E), weighted using the generalized plant action spectrum normalized at 300 nm (Cadwell, 1971; Aphalo et al., 2012), was 0.27–1.02 W m⁻² (4.86–18.36 kJ m⁻² d⁻¹). The distance between the plants and the lamp was 20 cm, and the pots were rotated each day on each bench, to minimize edge and position effects.

### Materials and Methods

**Plant materials and growing conditions**

Thirty plants produced vegetatively from three different Kalanchoe pinnata (RFA37.525, RFA39.958 and RFA39.963) matrix plants were planted in individual plastic pots (18 cm diameter and 15 cm high) filled with a commercial soil mixture (Nutriplan®). Prior to the UV-B treatment, these plants were grown on the campus of the Universidade Federal do Rio de Janeiro, under sunlight, with photosynthetically active radiation (PAR) varying from 400 to 800 μmol m⁻² s⁻¹ and the same watering system. The PAR was measured with a PAR sensor coupled to an FMS2 Hansatech fluorometer (Hansatech Instruments Ltd., King’s Lynn, UK). After 6 months, these plants of the same age were acclimatized in controlled-environment growth chambers for 2 weeks under the same PAR (200–400 μmol m⁻² s⁻¹, depending on plant position), temperature (32 ± 4 °C), manual irrigation (100 mL d⁻¹) and photoperiod (11.5 h of light). Plants were rotated every day, to minimize the effects of positional PAR. These basic growing conditions remained the same throughout the experiment.

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### Collecting and leaf anatomy

Anatomical analyses were performed on freshly developed simple leaves (third node) from both treatments (W and W + UVB). The first leaf collection started prior to the first UV-B supplementation (time zero – T0). Leaves of the plants from the W and W + UVB treatments were collected daily and fixed in FAA (Johansen, 1940) until the 11th day, and after that they were collected every 4 d, i.e. on the 15th, 19th and 23rd day (T15, T19 and T23, respectively). Leaf fragments were dehydrated and then embedded in Leica Historesin® and sectioned (10 μm) in a Spencer rotary microtome. Cross-sections were taken from the middle region of the petiole and in the middle third of the leaf blade. The sections were stained with Toluidine blue O (O’Brien et al., 1965) and mounted in Entellan®. Microchemical tests were performed on fresh material: Sudan III and IV to reveal lipids (Sass, 1951) and phloroglucinol to reveal lignin (Johansen, 1940). This fresh material was analysed by optical and fluorescence microscopy. For optical microscopy, an Olympus CH30 light microscope was used and the photographs were taken with an attached Olympus PM-C35B camera.

For fluorescence, a Leica DMLB microscope was used. Sudan IV was used in conjunction with a fluorescence I3 filter (blue light emission, bandpass 450–490 nm and 515 nm barrier filter) (Rittinger et al., 1987).

Leaf thickness measurements were made on cross-sections of leaves collected from three plants grown for 23 d under control and UV-B treatment. For each region and treatment, 15 measurements were taken on a Labomed microscope equipped with a digital camera and computer, used in conjunction with Pixel Pro® Image Analysis Software.

Statistical analysis was conducted with GraphPad Instat 3.0 for Windows®. Data were analysed with Student’s t-test ($P < 0.0001$).

### Results and Discussion

**Morphological differences between W and W + UVB plants**

Supplemental UV-B radiation induced morphological changes in the K. pinnata leaves. At the beginning of the experiment (T0), plants were morphologically identical.

However, as of the second day of the experiment (T2), the leaves of the W + UVB plants showed leaf curling in the direction of the adaxial side, mainly in the first and second node leaves (Fig. 1A). This photomorphogenetic response to the radiation has been observed in several other species (Barnes et al., 1996; Jansen et al., 1998; Zuk-Golaszewska et al., 2003; Boeger and Poulson, 2006). The leaf curling can decrease the leaf area exposed to the radiation and is due to a reduction in cell wall expansion by cells on the adaxial side, compared with those on the abaxial side (Wilson and Greenberg, 1993; Boeger and Poulson, 2006). Auxins appear to play a fundamental role in the phenomenon.
in this process. Indeed, this class of hormone has been shown to be the main substance that induces the common morphological changes in response to UV-B in plants (Jansen, 2002; Hectors et al., 2012).

Brown areas were noticed on the adaxial surface of the W + UVB leaves, especially those from the first and second nodes, but only in regions directly exposed to UV-B (Fig. 1B). During the UV-B exposure, these brown areas developed into a ‘brown film’ (Fig. 1C) on the leaf blade and petiole (Fig. 1D). Studies with field crops under UV-B radiation showed the occurrence of bronze or brown spots on leaves (Kakani et al., 2003), and these brown spots developed necrosis and chlorosis, and the leaves suffered desiccation and senescence. However, in these previous works there are no mentions of the foliar anatomy of these areas (Kakani et al., 2003). In our work, the K. pinnata leaves did not develop necrosis or chlorosis.

The apical bud and leaf margin buds (Fig. 1E, arrows) remained alive until the end of the experiment. Morphological changes were not observed on the abaxial side of the W + UVB leaves or in leaves from the W treatment through till the end of the experiment (T23) (Fig. 1F).

**Leaf anatomy**

At time zero (T0) the anatomy of the petiole (Fig. 2A) and the leaf blade (Fig. 4A) were similar in both W and W + UVB plants, with no differences from those previously described by Moreira et al. (2012).

On the second day of the experiment (T2),petioles of the W + UVB plants showed periclinal cell division (Fig. 2B, star) and loss of periclinal walls (Fig. 2B, asterisk) in some subepidermal regions of the adaxial surface. In addition, in some regions on the adaxial side there was a loss of epidermis (Fig. 2B, arrows). Anticlinal and periclinal divisions occupied more continuous regions in the course of the UV-B exposure.
from the fifth day onwards (T5) (Fig. 2C). At this time, the epidermis collapsed (Fig. 2C, arrows). As of the tenth day of the UV-B exposure (T10) these divisions began to form a wound-periderm (Fig. 2D). Below the layers of the pre-existing cells there are meristematic cell layers (phellogen) with rectangular, thin cells with thin walls, and the nuclei are clearly seen (Fig. 2D, ellipse). Above this layer, the recent cells formed by meristematic action, between the meristem and pre-existing cells, showed stronger suberin impregnation (phellem), as evidenced by Sudan IV observed under the fluorescence microscope using an I3 filter (Fig. 3A, arrow). Combining fluorescence microscopy analysis with Sudan IV, it is possible to observe the suberized cells in orange to red (Rittinger et al., 1987). Below the meristematic layer, there are more differentiated parenchymatic cells (Fig. 2D, asterisk), the phelloderm.

The microchemical tests showed negative results for suberin and lignin impregnation in phelloderm cell walls. The pre-existing cells of the more external layers differentiated thick walls, with suberin and lignin, evidenced by Sudan III and IV reagents and phloroglucinol.

As of the 19th day of the experiment (T19), the external layers of protective tissue (wound-periderm) became broken (arrow). (F) On the 23rd day of UV-B exposure (T23): lenticel-like structures (arrow) on the petiole adaxial side.

In the leaf blade, similar to the petiole, there was a loss and collapse of the epidermis on the adaxial side of the leaves on the second day (T2) of the experiment (Fig. 4B, arrow). However, the periclinal cell divisions in the adaxial sub-epidermal cell layers only occurred as of the fifth day of the experiment (T5) (Fig. 4C, stars). Also, besides the loss of...
chloroplasts on the adaxial side, there was a loss of epidermis papillae (Fig. 4C, arrow). As of the sixth day of the experiment (T6), anticlinal and periclinal divisions occupied more continuous regions and, as of the tenth day (T10), the wound-periderm began to be formed (Fig. 4D). The tissue constitution of the leaf blade (Fig. 4E) is similar to that of the petiole, with phellogen (Fig. 4E, ellipse), phellem (Fig. 3B, star) and phelloderm (Fig. 3B, asterisk). In all the newly formed tissue, rows of radial cells are produced by anticlinal divisions (Fig. 3B).

Towards the end of the experiment, the protective tissue had expanded (T23, Fig. 4F). Similarly for the petiole, the external layers of tissue broke down, but only at the end of the experiment (T23). At this time, a significant increase in leaf thickness could be seen (Table 2), mainly due to the production of phelloderm.

The petiole and leaf blade anatomy of the control plants (W) did not change throughout the experiment, and it was similar to what was described for the species in our previous study (Moreira et al., 2012) and similar to the leaf anatomy at time zero (T0) for the UV-B plants (Figs 2A and 4A).

The wound healing process in the leaf blade and petiole of Bryophyllum calycinum (syn. Kalanchoe pinnata) was previously studied by Welch (1945). The author made cuts in the leaves with a razor blade and noticed the production of suberized–lignified cells originated by the action of a meristematic layer in the wounded regions.

Rittinger et al. (1987) studied the healing process caused by wounding and infection in leaves of three different species (Mallus domestica Borkh., Prunus cerasus L. and Hordeum vulgare L.), but the authors did not observe wound-periderm formation in any of the studied species. On the other hand, the development of wound-periderm as a consequence of necrosis provoked by mechanical or pathogenic agents in plant organs, including leaves, has been known for a long time; such data can be found in Küster’s book (Küster, 1916). In fact, wound-periderm can be formed in response to wounds, irrespective of their nature (Ginzberg, 2008).

Wound-periderm formation is common in tubers and has already been described in response to γ-radiation in potatoes (Solanum tuberosum) (Thomas, 1982). The wound-periderm is produced in two stages; the first stage, immediately after wounding, is characterized by the generation of a suberized barrier referred to as a closing or sealing layer (Lulai, 2007). The second stage is the formation of a meristematic cell layer, which divides to produce suberized cells (Gizberg, 2008; Lulai et al., 2014). In our study, as of the second day of the experiment (T2) for the petiole and as of the fifth day (T5) for the leaf blade, periclinal cell divisions were observed in the sub-epidermal layers to form the phellogen, without a previous suberization of the outer cell layers. So, the formation of this wound-periderm in K. pinnata leaves seems to be different from that in potatoes and other species that follow this pattern.

UV-B radiation caused damage to the leaf blade and petiole epidermis [loss of epidermis and collapse of epidermal cells (Figs 2B, C and 4B, C)] as of the initial days of exposure to the radiation, and this could have triggered the wound-periderm formation.

The formation of a wound-periderm induced by UV-B radiation has not yet been reported in the literature. Varied responses to UV-B are related to different species genotypes and experimental conditions (Jansen, 2002; Xu and Sullivan, 2010; Robson et al., 2015), making the analysis complex.

Reported anatomical changes induced by UV-B, in general, include an increase in mesophyll, epidermis and wax cuticle thicknesses (Boeger and Poulsen, 2006; Fukuda et al., 2008; Mórsy et al., 2013), and many of them act as protection against radiation (Jansen et al., 1998; Jansen, 2002). These findings are consistent with ours, since we observed an increase in leaf thickness.

Conclusions

Kalanchoe pinnata is a species that is well adapted to arid regions, mainly due to its CAM metabolism, succulent leaves (Judd et al., 2009) and high phenolic composition. The wound-periderm-like tissue formed as a response to UV-B radiation can act as an additional ‘tool’ for protection of the species against radiation. The formation of such tissue in response to the administration of high doses of UV-B seems coherent. In conclusion, this study contributed to elucidate a leaf structural response to UV-B radiation, not yet reported in the literature and not common to this organ. This response is similar, but not equal, to that described previously by Welch (1945), triggered by cuts. The formation of wound-periderm-like tissue is probably an important acclimation response that K. pinnata has in order to survive in regions which are hot, dry and with high

Fig. 3. Microchemical tests of transversal sections from the petiole and leaf blade of K. pinnata cultivated during 23 d under supplemental UV-B radiation. (A) Test with Sudan IV on the petiole under a fluorescence microscope using an I3 band pass filter: stronger red colour in the walls of cells immediately above the meristematic tissue (arrow) reveals the stronger suberin impregnation when compared with cells above them (arrowhead). (B) Test with phloroglucionol reagent on a leaf blade observed under the optical microscope exhibiting a positive result for lignin impregnation in cell walls of the external cell layers.
Despite the absence of previous records of wound-periderm formation in response to UV-B radiation, it may be more widespread than we imagine. The brown areas seen in plants submitted to high sunlight or UV-B exposure should be analysed in order to verify their anatomical nature.

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LITERATURE CITED


