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Healing of *Gladioulus grandiflora* corms under refrigeration and *Fusarium oxysporum* infection

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Abstract

Background. The production and marketing of flower and ornamentals have increased in the world. However, about 50% of this production is lost due to injuries caused by precarious harvesting, transportation and storage, which facilitates tissue infection. The objective of this research was to characterize wound healing in refrigerated *Gladioulus grandiflora* corms and how this affects the entry and establishment of *Fusarium oxysporum* infection.

Methods. *Gladioulus grandiflora* corms were injured and stored at 12 ± 4 °C and a relative humidity of 90 ± 5%. Phenolic compounds, tissue darkening (melanin formation), lignification, suberization, resistance to infection, loss of fresh mass and respiratory rate were evaluated during healing of *G, grandiflora* corms.

Results. Injury to *G. grandiflora* corms caused cellular decompartmentalization and death, increasing the fresh mass losses and triggering oxidase activities by exposing enzymes to substrates and O_2.

Discussion. Cells adjacent to the wound underwent healing in a temporal sequence, starting with an increase in respiration rate, formation of carbon skeletons that increase the concentration of phenolic compounds used for the synthesis and deposition of lignin, melanin, and suberin in injured tissues.

Conclusion. These processes resulted in *G. grandiflora* corm healing by accumulation of lignin, melanin and suberin in wounded tissues, which sealed corm against water loss and *F. oxysporum* entrance by the third day after injury.

Key words: cork storage diseases; flowers; *Fusarium oxysporum*; ornamental horticulture.

Introduction

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Gladiolus stands out among floral productsfor its wide variety of having flower colors, high productivity, short production cycle, and high economic return (Usman et al., 2015). However, this plant is susceptible to diseases, such as fusariosis, which is the most severe and one of the most limiting diseases for its development (Shanmugam et al., 2017)and post-harvest life. Gladiolus corm susceptibility to *Fusarium* spp. (*Fusarium moniliforme*, *Fusarium oxysporum* f. spp., *Fusarium roseum* and *Fusarium solani*) is highly independent of the fungus species or gladiolus cultivar (Chandel & Deepika, 2010, Cordova-Albores et al., 2016).

Fusarium spp. persist in the soil for years as chlamydospores that can attach to corms in the post-production stage (Davies et al., 2006). Unhealed wounds caused during harvesting, handling, transport and storage facilitate *Fusarium* spp. infection (Gupta et al., 2016).

Plant wound healing involves a series of molecular and cellular eventsto repair injured tissues (Tisi et al., 2008). These events include callose biosynthesis, cell division, lignin and suberin layer formation, and production of structural proteins to protect plant tissuesby closing the wound site, minimizing dehydration, and reducing infection (Ibrahim et al., 2001).

The objective of the work presented here was to evaluate the healing events in *G. grandiflora* corms subjected to an injury that simulates those occurring under improper transport and handling conditions and to determine how it affects *F. oxysporum* infection and establishment under refrigerated conditions.

Material and methods

Raw material, healing induction and storage conditions- *Gladioulus grandiflora* Hort. corms were planted on Bioplant® substrate (Bioplant Agrícola Ltda., Nova Ponte, Minas Gerais, Brazil) in 900 mL pots and plants were grown in a greenhouse. Plants were irrigated when necessary

77 without fertilization. After 60 days, corms were harvested. The vegetative leaves and buds were
78 removed with a knife and the corms were washed to remove substrate residues. Half of the corms
79 were wounded superficially (2-3 times) by scraping with a steel brush (Sodomar 25 cm) one hour
80 after harvest and the remaining corms were used as unwounded controls. Wounded and
81 unwounded corms were stored 12 ± 4 °C and 90 ± 5% relative humidity for 21 d (Fugate et al.,
82 2016). The corms were evaluated after 0, 1, 2, 3, 4, 5, 6, 7, 14 and 21 d of storage.
83 **Tissue Darkening-** Tissue darkening was assessed by visual analysis (Fugate et al., 2016). The
84 images were captured with a Sony Cyber-Shot DSC-HX1 semiprofessional camera. The
85 predominant color in the image was captured in the program Paint - Windows 10, using the color
86 selection tool.
87 **Fresh weight loss-** Losses in fresh mass were obtained on individual corms using a MARK
88 31000 semianalytical balance with an accuracy of ± 0.01 g. The results were expressed as
89 percentage of fresh mass loss estimated by the equation: PMC= [(MFI - MFF) x 100]/MFI;
90 PMC= loss of corms mass (%); MFI= initial fresh mass loss (g) and MFF= final mass loss (g).
91 **Respiratory rate-** CO_2 produced by *G. grandiflora* corms was measured by titration. Onde
92 corms injured or not (control) were placed in hermetically sealed containers. A total of 10 mL of
93 0.5 N NaOH was placed in each vessel and, after 24 h, titrated with 1N HCl. The results were
94 expressed in mg of CO_2 100 g^{-1} of fresh matter. The respiratory rate was estimated by the
95 equation: mg CO_2 $100g^{-1}$ fresh matter= (B-L) x C/MF; B= volume in mL spent for titration of the
96 "control" (container without corm, only with NaOH); L= volume spent to neutralize NaOH; C=
97 correction factor of NaOH (0.98); MF= corms fresh mass at the evaluation time. The hourly
98 respiration rate was determined with the formula: mg CO_2 Kg^{-1} d^{-1}= mg CO_2 g^{-1} fresh matter x
99 1000/IT; IT= time interval between titrations (24h).

Lignification and suberization- Anatomical sections were manually removed from injured or unwounded (control) corm tissues with a razor blade. The sections were analyzed with UV epifluorescent light to detect polyphenols in suberin. Microscopy was performed using a HBO 50W (L2), a short-arc mercury lamp equipped with a G-365 excitation filter, a FT-395 chromatic beam splitter, and a LP-429 barrier filter. The images were captured on a Zeiss Axioskop 50 microscope (Jena, Germany) equipped with a Zeiss AxioCam color camera. Tissue sections were stained with 5 N HCL-saturated phloroglucinol and examined under standard light microscopy for lignification detection (Sabba & Lulai, 2002; Fugate et al., 2016). Lignification sites were identified by the specific reaction of the reagent with the coniferylaldehyde groups (Sarkanen & Ludwig, 1971) and cinnamaldehyde (Geiger & Fuggerer, 1979) resulting in an orange-reddish coloration.

Phenolic Compounds- One milligram of fresh tissue was placed in 10 mL of methanol: acetic: water solution (50:3.7:46.3), sonicated for 15 minutes, and centrifuged at 16,000 *g* for 15 minutes. An aliquot of the extract (0.2 mL) was taken and a 1:10 (v/v) solution of Folin-Ciocalte:water was added. This solution was incubated for 10 minutes at room temperature (Fu et al., 2010), and after this period a total of 0.8 mL sodium carbonate (7.5%) was added to the solution which was mixed and incubated for 30 minutes at room temperature. Concentrations of soluble phenolic compounds were determined by absorbance at 473 nm with gallic acid as standard.

Obtaining the *Fusarium oxysporum* isolates- *Fusarium oxysporum* isolates were obtained from Jaboatão maize plants in the Alagoa Nova municipality, Paraíba state, Brazil. Cultures were started with 100 mm x 15 mm mycelial fragment isolates placed in the geometric center of Petri dishes containing potato dextrose agar medium (PDA, Difco, Sparks, MD) and incubated at 25

°C for five days until agar was completely covered by fungi. After 0, 1, 2, 3, 4, 5, 6, 7, 14 and 21 days, the gladiolus corms were inoculated with a (10 mm diameter circular fragment of the fungal covered PDA plate. After inoculation, the corms were stored at 12 °C ± 4 °C and 90 ± 5% relative humidity for 30 days (Fugate et al., 2016). Disease severity in internal tissues was assessed at 30 days by excision and weighing of the infected tissue from each corm. The infection percentage was estimated by the total weight of the root and that of the infected portion.

Data analysis- The experiment was set up in a completely randomized design (CRD) with treatments composed of wounded or unwounded *G. grandiflora* corms with three replications. The data were tabulated in Excel 2016 (Microsoft, Redmond, Washington, EUA) and submitted to analysis of variance with SAS® (Software Business Analytics and Business Intelligence, Cary, North Caroline). Means were compared using the Tukey test at 1% of probability. Means and standard errors were plotted using SigmaPlot™ 10.0 program (Systat Software Inc. San Jose, California).

Results

Injuries resulted in transepidermal damage with collapse and cell death in *G. grandiflora* corms (Figure 1A). Physiological changes (increased mass losses and respiration) and biochemical changes (lignin, melanin and suberin deposition) occurred in the cells neighboring those killed by the wound. The wound caused gradual tissue darkening during storage, from brown to light brown (Figure 3).

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144 The fresh mass losses of wounded and unwounded *G. grandiflora* corms was similar in
145 the first three days of storage and higher in the injured ones, from the fifth (8.2%) to the twenty-
146 first day (28.4%) storage (Figure 2A).

147 Respiration rate of the injured *G. grandiflora* corms was higher between the first and fifth
148 day, lower on the twenty-first day and similar to the control on the sixth to fourteenth day of
149 storage. The respiration rate in the control was relatively constant during storage (Figure 2B).

150 The concentration of phenolic compounds in injured *G. grandiflora* corms increased
151 during the three days after the injury, with greatest concentrations occurring on the third day in
152 storage. Phenolic compounds were not detected during storage in uninjured corms (Figure 2C).

153 Suberization preceded lignification. The formation of a thin, continuous suberin layer was
154 observed, on the third day, in the cells at the wound site. Fluorescent parallel layers of cells were
155 observed below the wound surface, from the seventh to the twenty-first day after injury, forming
156 an extensive continuous layer of suberin. Isolated, irregular and discontinuous lignification sites
157 were observed along the outer (intercellular) cell wall and adjacent to the inner (intracellular)
158 wall on the third day after injury. During the subsequent days, lignin became thicker and
159 continuous on the cell walls of some wound cells, but without forming a continuous layer which
160 would close off the wound site (Figure 3).

161 Wounding of *Gladioulus grandiflora* corms was associated with a 12% increase in corm
162 infection by *F. oxysporum* relative to unwounded controls on the first day after injury and
163 resistance to infection improved in cormsthree days after injury, althoughinfection increased
164 from the fourth to the seventh day of storage in injured corms, remained high until the fourteenth
165 day and declined on the twenty-first day of storage. Infection in uninjured corms was restrict to
166 the outer layers of the corm and did not penetrate the internal tissues (Figure 4).

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Discussion

The cell collapse and death in injured corms, is due to the rupture and opening of the cell wall and plasma membrane, resulting in the decompartmentalization and leaching of the cellular content (Silva et al., 2010) similar to that reported for *Arabidops*is (Cutler & Somerville, 2005), whose cell collapse caused by injuries, resulted in their death. Cell collapse and death occur due to loss of the integrity of the wall and plasma membrane, flooding the cytosol with ions that imbalance cellular functions (Lemasters et al., 1987; Schapire et al., 2009). Cell death may impair the plant tissue health (Jones, et al., 2016) and to avoid this, living cells near experiences physiological (increased loss of mass and respiration) and biochemical changes (deposition of lignin, melanin and suberin) to heal injured tissue (Lulai, Suttle & Pederson, 2008). This was reported for *Arabidopsis*, whose cells near those killed by the injury, had rapid protein aggregation to aid in healing tissues (Cutler & Somerville, 2005). These physiological and biochemical changes were induced in *Beta vulgaris altissima* L. (Fugate et al., 2016), *Solanum tuberosum* L. (Edgell et al., 2008), *Beta vulgaris esculenta* L. (Schouten & van Schaik, 1980) and *Daucus carota* L. (Satoh et al., 1992) rendering injured tissue relatively impermeablethereby limiting water loss and microbial invasion (Ibrahim et al., 2001). The gradual darkening of damaged tissue is due to melanin formation resulting from the decompartmentalization and the cellular content leaching that exposed enzymes to substrates and O_2, as reported for sugar beet, which also experienced tissue darkening during healing (Ibrahim et al., 2001; Fugate et al., 2016). This darkening, during healing, is presumably due the oxidation of colorless phenolic compounds to yellow/red *o*-quinones by peroxidase (POD) and polyphenoloxidase (PPO) action (Busch, 1999, Ibrahim et al., 2001). *O*-quinones spontaneously react with themselves or with

proteins, amino acids and phenolic compounds to form melanin, an insoluble brown-colored polymer (Rouet-Mayer et al., 1990). POD induction in *B. vulgaris altissima* wounded (Bernards et al., 1999) and roots (Fugate et al., 2016) coincided with melanin formation, indicating that these enzymes are probably involved in the tissue darkening during the gladiolus corm healing.

The similarity in the fresh mass losses between injured or uninjured corms in the first three days of storage was due to the relative humidity gradient between air and wounded tissues after leaf and root hairs removal from all corms. The high fresh mass losses in the first days after root and tuber harvesting is common and it was observed in *Beta vulgaris altissima* L. (Wiltshire & Cobb, 2000; Fugate et al., 2016) and *Solanum tuberosum* L. (Lulai et al., 2008), being attributed to common wounds during collection, stacking and handling. The greater fresh mass losses in injured corms from the fifth to the twenty-first day of storage indicates tissue inability to avoide water losses by incomplete wound healing. The fresh mass losses in plant organs is a parameter used to evaluate wound healing (Bajji et al., 2007; Lulai et al., 2008) and it was used to determine *S. tuberosum* (Soliday et al., 1979; Schreiber et al., 2005; Lulai et al 2008) and *Ipomoea batatas* (L.) Lam (van Oirschot et al., 2006) and *B. vulgaris altissima* (Fugate et al., 2016) what were considered complete healing when water loss was reduced by the accumulation of hydrophobic barriers (suberin and lignin) (Lulai et al., 2008).

The highest respiration rate in injured corms in the first five days of storage is related to the metabolic energy and substrate needed as substrates for biopolymer biosynthesis to aid in defense and regeneration. These are synthesized from carbon intermediates from glycolysis, the oxidative pathway of the pentoses phosphate and the mitochondrial tricarboxylic acid cycle. These pathways intensify in response to injury and other biotic and abiotic stresses (Naczk & Shahidi, 2004; Vanlerberghe, 2013) to avoid oxidative damage and regenerate tissues. The

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213 respiration rate increase is possibly due to demand for metabolic energy and substrates for the
214 healing processes as found in *B. vulgaris altissima* (Fugate et al., 2016) and other higher plants
215 (Lipetz, 1970). Compounds and biopolymers involved in tissue defense and regeneration include
216 phenolics, phenylpropanoids, specific fatty acids (Bernards, 2002; Lulai, 2007) and suberin
217 polyphenols (Borg-Olivier & Monties, 1993), the principal compounds accumulated in potato
218 tubers and its oxidation implies suberization (Bernards et al., 1999). Similar respiration of the
219 injured corms between the sixth (330 mg CO_2 kg^{-1}) and the fourteenth (297 mg CO_2 kg^{-1}) days
220 indicates the stabilization of the substrate production for the biosynthesis of compounds and
221 biopolymers of defense and tissue regeneration as reported for *B. vulgaris altissima* (Klotz et al.,
222 2009; Megguer et al., 2017). Respiration rate stabilization or reduction is used to determine
223 wound healing in plant tissues, since increased respiration is directly related to cellular
224 mechanisms to maintain metabolic homeostasis during stressg (Vanlerberghe, 2013). The
225 respiration rate decrease in *B. vulgaris altissima* was directly related to healing (Lipetz, 1970;
226 Lafta & Fugate, 2011; Fugate et al., 2016). The lower respiration rate in injured corms on the
227 21st day of storage is due to the decrease in the density of live cells caused by collapse and cell
228 death, what is necessary for tissue respiration (Lulai, Suttle & Pederson, 2008).

229 The increase in the phenolic compound concentration in injured corms, with peak on the
230 third day, may be due to the increase in respiration rate providing additonal carbon intermediates
231 for their biosynthesis. An increase in respiration rate and increased concentration of phenolic
232 compounds were reported for potatoes during wound healing (Bernards et al., 1999). The
233 increase of these compounds resulted in incremental lignin and suberin biosynthesis, that
234 coincided with higher peroxidase (POD) activity (Bernards et al., 1999). Suberin biosynthesis
235 depends on phenolic compound accumulation (Kolattukudy, 1981), whose concentration is

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236 associated with injuries (Reyes et al., 2007). An increase in phenolic compound concentration
237 increase has also been reported after injury to sugar beet and carrot roots (Torres-Contreras et
238 al., 2014; Becerra-Moreno et al., 2015; Fugate et al., 2016).

239 D5: Suberization, before lignification, in *G. grandiflora* injured corms can be explained by
240 the hydroxycinnamic acid predominance in cell walls, as reported for *Clivia miniata* (Schreiber,
241 1996; Zeier & Schreiber, 1997), *Quercus suber* (Gil et al., 1997) and *Solanum tuberosum*
242 (Bernards et al., 1995; Negrel et al., 1996). Hydroxycinnamic acid is the only polyphenolic
243 component found in suberized cells that is not found in lignified cells. (Lewis & Yamamoto,
244 1990). The formation of a thin and continuous suberin layer in the injured corms shows that
245 suberization was more important than the lignification for closing off wound sites in G.
246 grandiflora corms since continuity is more important than thickness (van Oirschot et al., 2006).
247 The induction kinetics of healing events in *Arabidopsis* roots peaked two days after injury,
248 related to increased suberization (Domergue et al., 2010). The lignification occurred in isolated,
249 irregular and discontinuous sites in the cell walls of those bordering the wound, indicating low
250 efficiency as a hydrophobic barrier and protection of the tissues injured similar to our results.
251 This suggests that lignin is not one of the main contributors to the wounds healing in gladiolus
252 corms. Differences in lignin and suberin biosynthesis are still unknown (Bernards et al., 1999;
253 Fugate et al., 2016), but includes the induction of phenylalanine ammonia (PAL) by injury,
254 associated with phenylpropanoid biosynthesis and biosynthesis of specific fatty acids (Bernards,
255 2002; Lulai, 2007). Inhibition of PAL activity reduced the accumulation of suberin in injured
256 potato tissues (Lulai et al., 2009), suggesting that this enzyme is directly related to the suberin
257 accumulation in tubers. The sweet potato scarification differs between varieties, temperature and
258 relative humidity during storage (Walter & Schadel, 1983; van Oirschot et al., 2006) with the

formation of a thick lignin layer (17 cell layers) at 60% RH and another thin (4-6 cell layers) in roots maintained at 95% RH (Strider & McCombs, 1958). In sugarbeet roots suberization preceded the lignification at 12 °C (Fugate et al., 2016) and lignification preceded and exceeded suberization in the healing process of stored beet roots (Ibrahim et al., 2001).

Greatest infection of injured corms by *F. oxysporum* on the first day after injury was likely due to loss of the outer protective layers of the corm by the collapse and death of the cells that received the wound directly. Improved resistance of injured cormse after the third day post-injury is likely due to deposition of suberin/lignin, accumulation of melanin or other antifungal substances, as reported during root healing of *D. carota* (Davies & Lewis, 1981) and *S. tuberosum* (Kolattukudy, 1987; Lulai & Orr, 1994; Lyon, 1989; Lulai & Corsini, 1998). However, increases in infection increasing from the fourth to the seventh day of storage in injured corms is difficult to explain but may indicate that suberization and lignification layers were insufficient to provide for durable resistance against this pathogen during storage. Another possibility is that after 3 d, injured corms were dehydating more rapidly than uninjured corms. Could the dehydration make them more susceptible to disease (this occurs in beets). The durability of *E. carotovora* subsp. *carotovora* resistance in injured potato tubers of the 'BelRus' variety was explained by the segmented deposition of suberin within each cell layer, first on the external tangential cell walls followed by the radial walls and then on the inner tangentials. This was not observed for tubers of the 'Superior' variety, indicating that the absence of segmented deposition was responsible for the development of resistance to *E. carotovora* subsp. *carotovora* (Lulai & Corsini, 1998).

Conclusion

Wounding lead to increased water loss, increased accumulation of phenolic compounds and transient increases in respiration rate. The defense mechanism induction results in the *G. grandiflora* corm wound healing by the lignin, melanin and suberin deposition and accumulation in the tissues damaged. Wound-healing was evident by the 3rd day after injury as suberized cell layers formed at the wound site and signs of lignification were found at this time. These wound-healing processes provided some resistance against infection, although overall, injured corms were more susceptibile to infection than unwounded corms.

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Figure 1

Cross-sectional view of *Gladioulus grandiflora* corm injury.

Cross-sectional view of *Gladioulus grandiflora* corm injury. Arrows indicate cell wall rupture and opening and plasma membrane (Objective 20x) (A). Simplified scheme of darkening reactions and melanin formation after wounding (B).

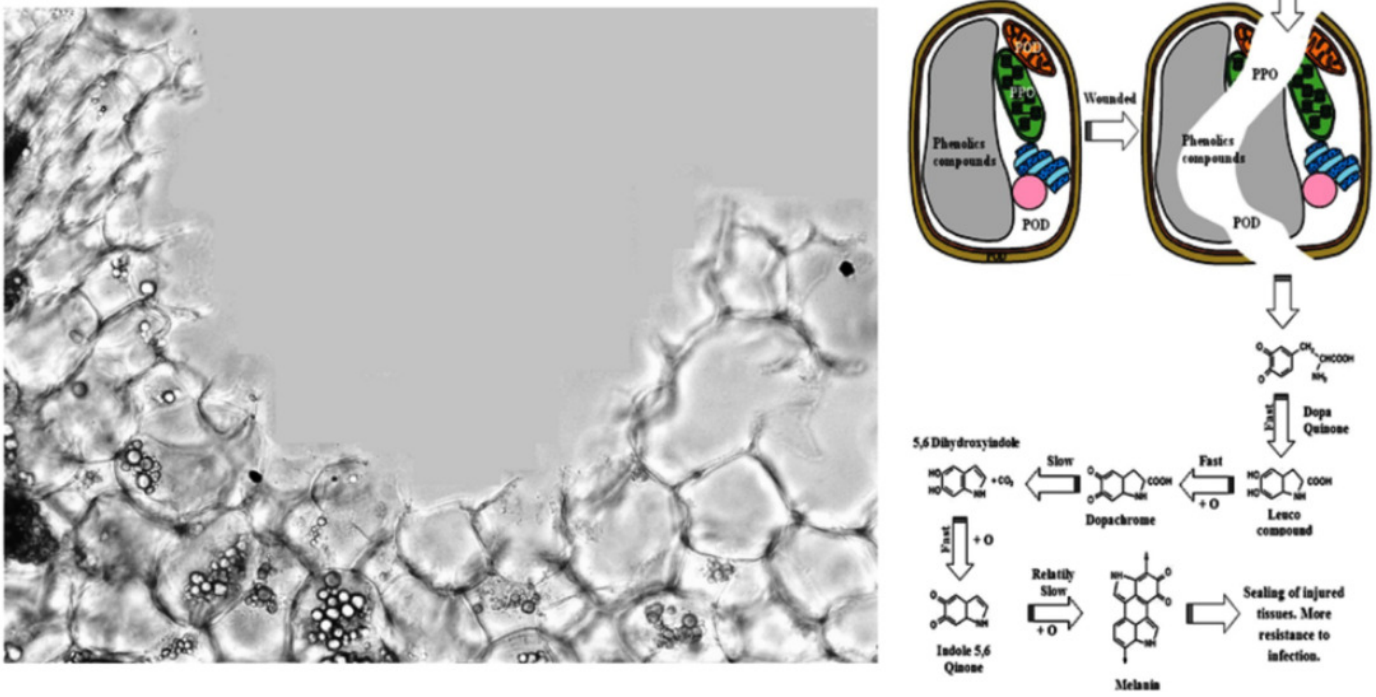

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Figure 2

Fresh weight losses, respiratory rate and phenolic compounds of *Gladiolus grandiflora* corms injured or not.

Fresh weight losses (%) (A); respiratory rate (mg CO_2 Kg^{-1} h^{-1}) (B) and phenolic compounds (mg gallic acid g^{-1}) (C) of *Gladiolus grandiflora* corms injured or not and storage at 12 °C for 21 days.

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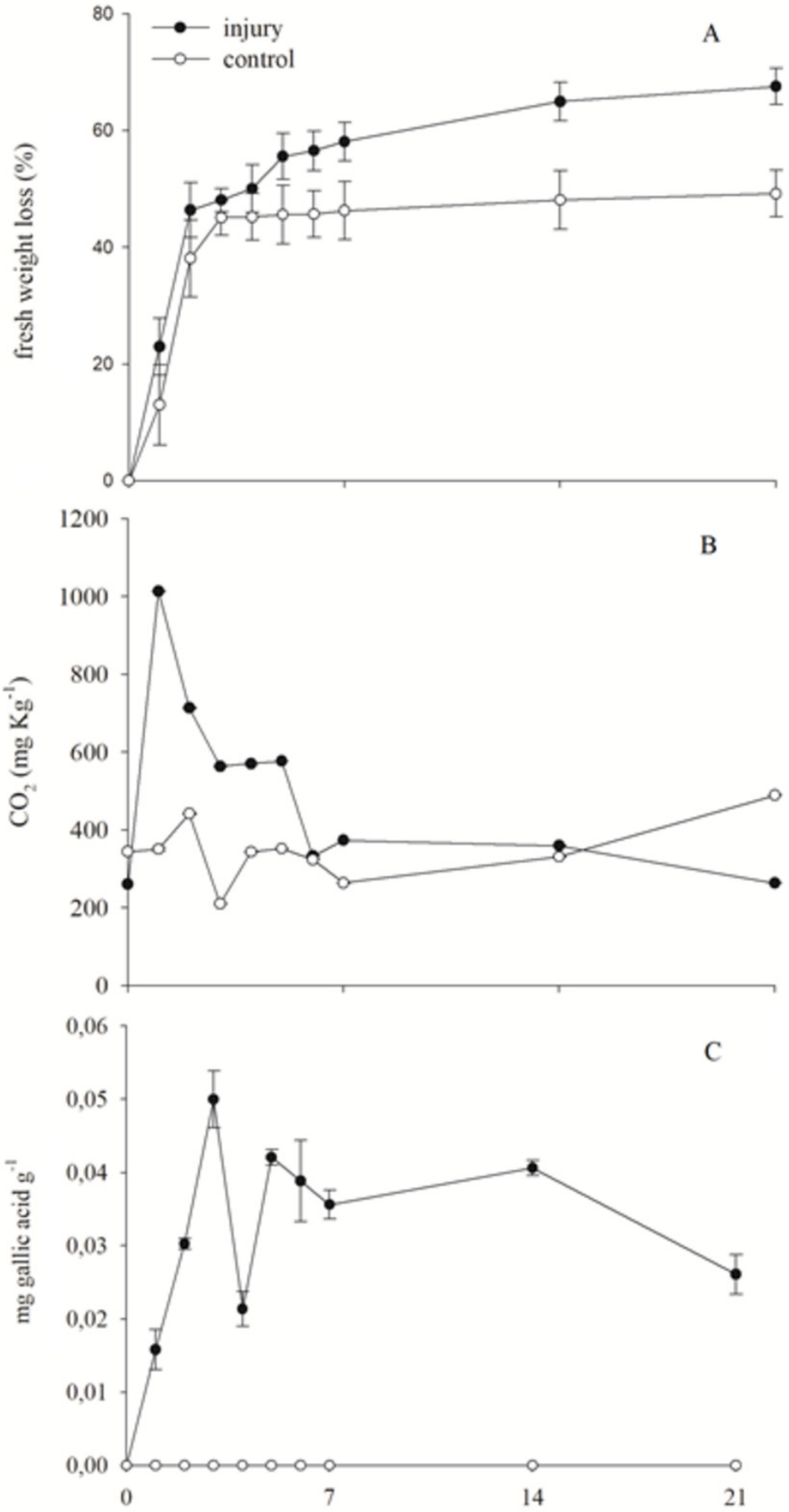

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Figure 3

Suberization, lignification and surface discoloration in *Gladioulus grandiflora* corms injuried

Suberization (100x), lignification (200x) and surface discoloration in *Gladioulus grandiflora* corms injuried and stored at 12 °C for 21 days. The arrows identify areas lignified. Bar= 100 µm.

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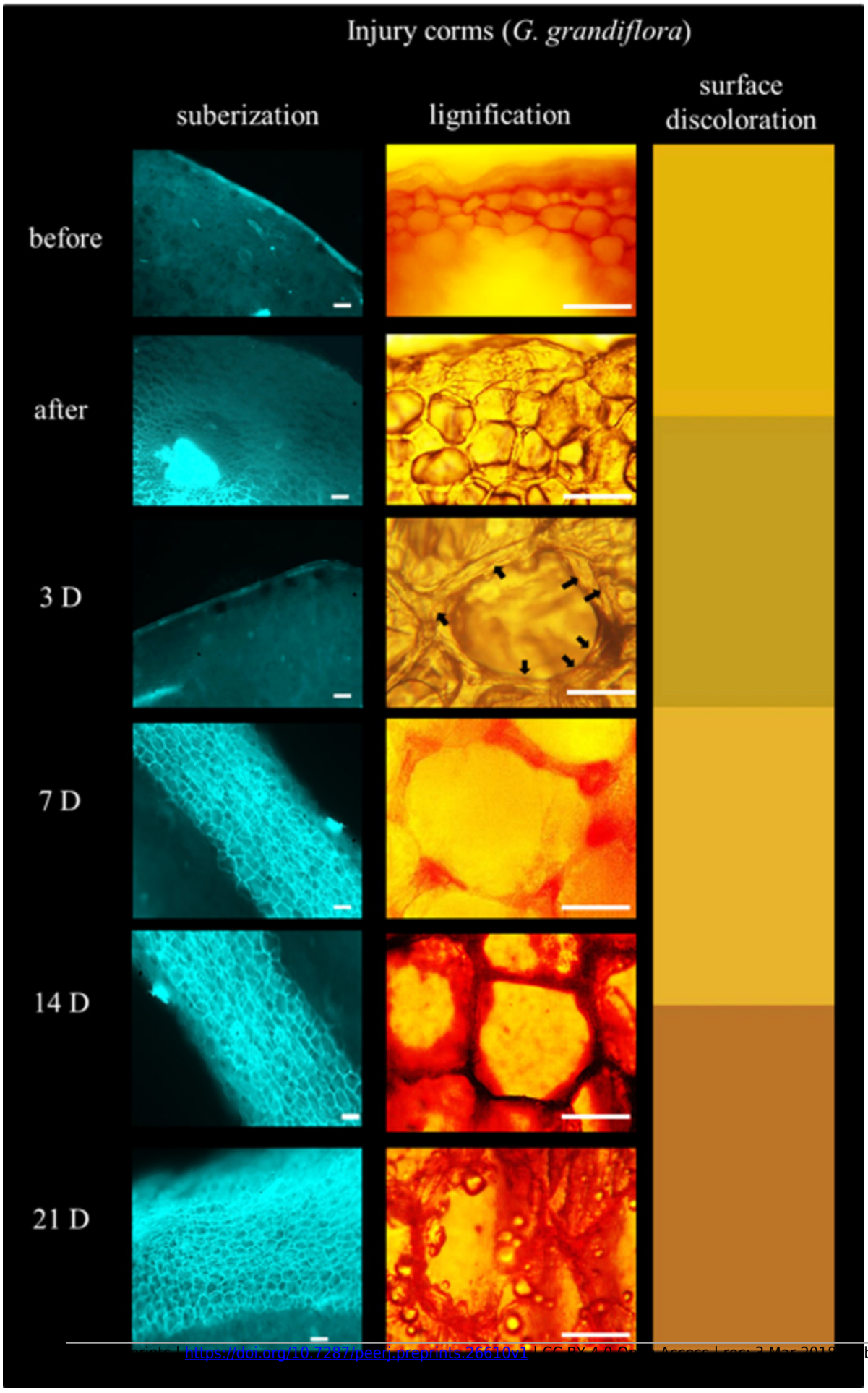

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Figure 4

Infected tissue of *Gladiolus grandiflora* corms by *Fusarium oxysporum*

Infected tissue (%) of *Gladiolus grandiflora* corms stored at 12 °C and 90% relative humidity after 30 days.