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ORIGINAL ARTICLE

Spatio-temporal relief from hypoxia and production of reactive oxygen species during bud burst in grapevine (*Vitis vinifera* L.)

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ABSTRACT

- Background and Aims

Plants regulate cellular oxygen partial pressures (pO_2), together with reduction/ oxidation (redox) state to manage rapid developmental transitions such as bud burst after a period of quiescence. However, our understanding of pO_2 regulation in complex meristematic organs such as buds is incomplete, and particularly lacks spatial resolution.

- Methods

The gradients in pO_2 from the outer scales to the primary meristem complex were measured in grapevine (*Vitis vinifera* L.) buds, together with respiratory CO_2 production rates and the accumulation of superoxide and hydrogen peroxide, from ecodormancy through the first 72 h preceding bud burst, triggered by the transition from low to ambient temperatures.

- Key Results

Steep internal pO_2 gradients were measured in dormant buds with values as low as 2.5 kPa found in the core of the bud prior to bud burst. Respiratory CO_2 production rates increased soon after the transition from low to ambient temperatures and the bud tissues gradually became oxygenated in a patterned process. Within 3 h of the transition to ambient temperatures, superoxide accumulation was observed in the cambial meristem, co-localising with lignified cellulose associated with pro-vascular tissues. Thereafter, superoxide accumulated in other areas subtending the apical meristem complex, in the absence of significant hydrogen peroxide accumulation, except in the cambial meristem. By 72 h, the internal pO_2 gradient showed a biphasic profile, where the minimum pO_2 was external to the core of the bud complex.

- Conclusions

Spatial and temporal control of the tissue oxygen environment occurs within quiescent buds, and the transition from quiescence to bud burst is accompanied by a regulated relaxation of the hypoxic state and accumulation of reactive oxygen species (ROS) within the developing cambium and vascular tissues of the heterotrophic grapevine buds.

KEYWORDS

Bud burst, *Vitis vinifera* L., Grapevine, Reactive Oxygen Species, Superoxide, Hypoxia, Oxygen Partial Pressure, Meristem, Development, Respiration, Ecodormancy, Quiescence.

INTRODUCTION

The buds of perennial trees and vines comprise one or more embryonic shoots with multiple meristems of diverse organogenic states, enclosed in a protective shell of dense scales. Similar to germinating seeds, the transition from quiescence to metabolically-active occurring during bud burst is rapid, and requires the re-structuring of intercellular communication, respiratory and biosynthetic metabolism and cell division and expansion. The identity, pluripotency and fate of cells in the meristem is determined by spatial organisation (Esau, 1977, van den Berg et al., 1995), which is compounded in the embryonic shoot. Hence, this transition requires intricate spatial and temporal coordination of intercellular signalling networks within and between the functional domains of each meristem.

Oxygen is an essential substrate and signal in all aerobic organisms. Plants regulate the availability of oxygen and its metabolism during key transitions, including the regulation of quiescence (Considine and Foyer, 2014). Within this context the cellular reduction/ oxidation (redox) hub plays a key role (Gapper and Dolan, 2006, Considine and Foyer, 2014), and we suggest the partial pressure of oxygen (pO_2) also plays an important role, as known in animals and other aerobic organisms (Brahimi-Horn et al., 2007). The complex roles of redox processes in seed germination (Diaz-Vivancos et al., 2013 and references therein) and the control of pO_2 are far from understood (Bradford et al., 2008, Borisjuk and Rolletschek, 2009). Similarly, our current knowledge of redox and pO_2 sensing and signalling during bud burst is limited, particularly in terms of the spatial resolution of oxygen dynamics. Animal stem cell models consider that the redox environment, together with hypoxia (low pO_2), are central regulators of the stem cell niche, that are key to cell identity and the maintenance of quiescence and pluripotency (Mohyeldin et al., 2010, Wang et al., 2013). The quiescent centre of the root meristem

resides in an oxidised niche (Jiang et al., 2003, Jiang and Feldman, 2005). It is probable that the organising centre and stem cells of the shoot apical meristems have similar requirements (Reichheld et al., 2007, Considine and Foyer, 2014).

In plants, as in animals, intracellular redox signals govern the cell cycle (Colucci et al., 2002, Jiang et al., 2003, Rothstein and Lucchesi, 2005, Diaz Vivancos et al., 2010). The local perception of pO_2 in animals enables acclimation during developmental transitions, as well as mediating responses to various stress conditions and pathologies (Brahimi-Horn et al., 2007). Recent studies have increased understanding of the sensing and signalling of pO_2 in plant oxygen-stress responses (Gibbs et al., 2011, Licausi et al., 2011). However this type of regulation has scarcely been studied in developing systems other than seeds.

Regulation of respiration is central to the transition from quiescence to the metabolically active state. During seed germination or bud burst, respiration increases because of the requirement for oxidative phosphorylation and reducing power (Morohashi and Shimokoriyama, 1975, Hourmant and Pradet, 1981, Bewley, 1997). Studies on seeds have demonstrated a regulatory role of redox signalling during germination and clear spatial gradients that illustrate the function of reactive oxygen species (ROS) and low molecular weight antioxidants in cell division and expansion (Gidrol et al., 1994, Schopfer et al., 2001, Oracz et al., 2009, Kranner et al., 2010, Rewers and Sliwinska, 2014).

The transition to bud burst can be accelerated by numerous sub-lethal stresses, including transient inhibition of respiration, heat shock or hypoxia (Esashi and Nagao, 1973, Erez et al., 1980, Erez, 1987), as is also the case with seed germination (Roberts, 1962, Siegel et al., 1962, Siegel et al., 1964, Chen, 1970, Al-Ani et al., 1985). ROS are proposed to be key signalling agents induced by respiratory inhibition, as they function

both directly on the cell cycle and by modulating activities of plant growth regulators such as ethylene, abscisic acid and auxin (Ophir et al., 2009). This fits with earlier suggestions that repressed catalase activity (Shulman et al., 1983, Nir et al., 1986) and increased production of hydrogen peroxide stimulate bud burst in grapevine (Perez and Lira, 2005, Vergara et al., 2012a). Indirect evidence that dormant buds reside in an hypoxic state comes from analyses of gene expression. Transcripts encoding proteins involved in oxidative phosphorylation and the TCA cycle are repressed in dormant buds while those encoding components involved in glycolysis, pyruvate metabolism, fermentation and redox networks are increased (Halaly et al., 2008, Ophir et al., 2009, Vergara et al., 2012b). Much of these data come from buds under stress conditions.

The scales of buds have low oxygen permeability and so the enclosed tissues are likely to be hypoxic, similar to the situation in dry seeds (Borisjuk and Rolletschek, 2009). In the seeds of some species, the suberised cell layers beneath the seed coat act as a barrier to oxygen diffusion, and their removal accelerates germination (Collis-George and Melville, 1974, Rolletschek et al., 2007). To date, no studies in the literature report data on pO_2 values in buds. The following studies were therefore performed to resolve this issue, and to examine the cellular redox poise and pO_2 status during bud burst. Furthermore, we aimed to resolve the spatio-temporal changes in these parameters that accompany the transition to bud burst, in a simplified developmental system that may provide a platform for further studies in a range of conditions and quiescent states (Considine and Foyer, 2014). The following experiments were performed on grapevine (*Vitis vinifera* L.), which is one of the most economically important woody perennial crop species, and has become a model species for research on perennial woody plants. Due to the anatomical complexity of the grapevine bud relative to other meristematic organs, it is useful to describe grapevine bud structure (Pratt, 1974, May, 2004). The

mature bud complex, or N+2 according to May (2004), comprises a hierarchy of three buds – primary, secondary and tertiary, each resembling primordial shoots (**Fig. 1**). The primary bud is the most developed and by maturity bears 12-15 nodes, including inflorescence, tendril and leaf primordia, enclosed by layers of bracts and hairs. During maturation prior to winter, outer bracts lignify and harden to physically protect the bud over winter. Concurrent with this is a gradual cessation of meristematic activity and the acquisition of tolerance to desiccation and chilling (Schrader et al., 2004, Rohde et al., 2007, Ruttink et al., 2007). The cessation of growth involves the acquisition of dormancy, defined as the failure of an intact, viable bud to burst in otherwise conducive conditions, until repressive factors are overcome through entrainment to seasonal signals such as chilling and photoperiod (Bewley, 1997), otherwise known as endodormancy (Lang et al., 1987). Once endodormancy is overcome, the bud is said to be ecodormant, i.e. quiescent but awaiting conducive conditions for growth. In this study, we refer to the mature bud complex as a whole, although pO_2 measurements were directed at the primary bud, and the secondary and tertiary buds were often lost during histological processing. The data presented here show that ecodormant buds undergo a regulated transition from hypoxia to the oxygenated state during bud burst. These findings provide a platform to further explore and dissect the roles of these signalling agents in mediating transitions in bud dormancy governed by environmental and developmental inputs.

MATERIALS AND METHODS

Plant material

Grapevine var. Crimson Seedless canes with mature dormant buds were harvested mid-winter from a vineyard in Yallingup Siding, Western Australia (Latitude: -33.694; Longitude: 115.102). Canes with buds intact were stored at 4 °C in the dark until they had received at least 5500 chilling hours (*c*. 7 months). The low degree of quiescence of the buds after cold-storage was confirmed by growing single-node cuttings of nodes 5-7 (explants, numbered acropetally) at 23 °C in vermiculite in darkness, with water maintained at field capacity (refer **Fig. 1** for developmental progression). Nodes 5-7 were chosen due to positional effects noted previously (Antcliff and May, 1961). The cumulative rate of bud burst was scored similarly to that described by Antcliff and May (1961) and according to the modified Eichorn-Lorenz scale (EL; Coombe, 2004), showing that 50 % of buds had reached EL-4 after 96 h at 23 °C and 80 % bud burst by 240 h (data not shown). On this basis we chose to study a time series over 72 h from transfer to 23 °C, in continuous darkness to minimise complexity. One or more single nodes were considered a biological replicate, as described for each assay.

Internal O_2 partial pressure (pO_2)

The internal pO_2 of buds were measured after 3, 24 and 72 h at 23 °C, using a Clark-type oxygen microelectrode with tip diameter of 25 µm (OX-25, Unisense A/S, Aarhus, Denmark). Internal pO_2 was also measured in buds with the outer scales removed by scalpel 10 min prior, after 3 h at 23 °C. Microelectrodes were calibrated at atmospheric pO_2 (20.87 kPa) and at zero O_2, then mechanically guided into the buds, from the outer scale surface to the core of the primary bud, in 25 µm steps to a depth of 2000 µm using motorised micro-manipulator (MC-232, Unisense). The microelectrode recording was

allowed to stabilise for 20 s after each step with measurements taken over the subsequent 10 s. Means and 95 % confidence intervals of individual buds (n = 3) were calculated using R (R Development Core Team, 2014) and graphics were compiled using the latticeExtra package and functions within (Sarkar and Andrews, 2013).

Bud respiratory CO_2 production

Four buds per biological replicate were excised from the cane by transverse section at the base of the bud, weighed and placed onto thin agar plates, cut-side down, so that O_2 entry and CO_2 exit would occur across the bud scales rather than via the cut base. The rate of CO_2 production of each biological replicate was measured in the dark, in an insect respiration chamber (6400-89, Li-COR, Nebraska, Canada) attached to Li-6400XT portable gas exchange system. Measurements were performed at 23 °C, in CO_2-controlled air (380 µmol CO_2.mol^{-1} air) with 100 µmol.m^{-2}.s^{-1} air flow, at 55-75 % relative humidity. The system was allowed to stabilise for 10 min before recording and until the "stableF" value was equal to 1, i.e. the condition of humidity, CO_2 and air flow were in equilibrium and stable. Means and 95 % confidence intervals were determined by fitting the time-series of CO_2 evolution to a quadratic equation of the form, $y = \alpha + \beta_1 x + \beta_2 x^2$, using the linear model function within R (R Development Core Team, 2014) and plotted using ggplot2 (Wickham, 2009).

Histology

Chemicals for histology were supplied by Sigma (St. Louis, USA) unless otherwise stated. To confirm the path of the pO_2 microelectrode, buds were fixed for sectioning immediately after measurement. Before excision and fixation, a vector was cut in a sagittal plane from each side of the bud complex, adjacent to the primary bud and parallel to the path of the microelectrode to aid penetration of the fixative. Buds were

then excised from the cane by transverse section at the base of the bud, then fixed in 10 % v/v formaldehyde (Chem-Supply, Adelaide, Australia) with 5 % v/v propionic acid (Ajax Chemicals, Sydney, Australia) overnight at 4 ºC, and subsequently dehydrated in serial ethanol solutions (15, 20, 25, 30, 50, 75, 90 and 100 % v/v), 30 min each, with gentle agitation at 4 °C. Buds were then embedded in paraffin wax. Sagittal sections (5 μm) of the bud were made on a microtome (Leica Biosystems, RM2255, Nussloch, Germany), transferred to slide, de-waxed and stained with 0.05 % w/v of toluidine blue O in 0.1 M phosphate buffer pH 4.8. The sections were then scanned at 20x magnification by Aperio Scanscope LX (Leica Biosystems).

Histological detection of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) were performed on bud sections from explants grown for 0, 3, 23 or 72 h at 23 °C. The methods of Groten et al. (2005) were followed with minor change: nitrobluetetrazolium (NBT) and 3,3'diaminobenzedine (DAB) were each dissolved in 10 mM phosphate buffer pH 7.8 without dimethylsulfoxide. Buds were excised from the cane as described to visualise the path of the microelectrode, and stained under light vacuum for 8 h at room temperature in darkness. Stained buds were fixed in 4 % v/v formaldehyde (Chem-Supply) in a buffer of 5 mM $MgSO_4$, 5 mM EGTA and 50 mM PIPES pH 6.9, vacuum infiltrated for 1 h, incubated overnight at 4 °C, dehydrated in serial ethanol solutions (15, 20, 25, 30, 50, 75, 90 and 100 % v/v), 30 min each, with gentle agitation at 4 °C. The buds were then transferred into 1:1 v/v of ethanol: Steedman's wax solution (Norenburg and Barrett, 1987) and incubated for overnight at room temperature prior to embedding. Serial sagittal sections of the bud were made at 20 μm intervals using a microtome (Leica Biosystems, RM2255), transferred to slide and de-waxed in 100 % followed by 50 % v/v, 5 min each solution. The sections were then scanned at 20x magnification by Aperio Scanscope LX (Leica Biosystems).

To visualise lignin, NBT-stained buds were counter-stained with 0.05 % w/v Auramine-O (Ajax Chemicals, Auburn, Australia) in deionised water. A drop of stain solution was placed on each section and let to absorb for 1 min before washing the slides with sprayed water. The stained sections were then visualized using Carl Zeiss microscope (D-708Z Oberkochen, West Germany) with blue light 450 - 490 nm.

RESULTS

CO_2 production and internal oxygen partial pressure (pO_2)

Respiratory CO_2 production rates increased from *c*. 4.0 to 5.2 nmol CO_2.g $FW^{-1}.s^{-1}$ in ecodormant buds maintained at 23 °C over the first 72 h following the transition from low to ambient temperatures. Subsequently respiration rates fell to 4.0 nmol CO_2.g $FW^{-1}.s^{-1}$ by 144 h (**Fig. 2**), showing that metabolic activity was increased upon transition to conducive growth conditions for bud burst.

We determined the internal pO_2 profile from the outer scale towards the core of the primary bud complex; At 3 h after transfer to 23 °C, which was the earliest stage of measurement, the internal pO_2 was hypoxic immediately within the scale (*c*. 10 kPa *cf*. air = 20.6 kPa), declined towards 5 kPa within the outer 500 μm and declined steadily to *c*. 2.5 kPa through to the core of the bud complex (**Fig. 3A**). Some replicate data showed indetectable O_2 (severe hypoxia/potential anoxia) at the core. Removal of the outer layer of scales at this time point resulted in oxygenation of the outer 15-1800 μm of the tissue profile, relative to the intact bud, however the core remained near 2.5 kPa (**Fig. 3B**). Despite this effect, de-scaling buds had no significant effect on the rate or completion of bud burst to stage EL-4, relative to intact buds (data not shown, refer Materials and Methods). We then determined the pO_2 profiles of intact buds at 24 and 72 h after transfer to 23 °C to determine whether removal of the scale at 3 h simply expedited the normal progression of oxygenation within the bud. By 24 h, only the pO_2 of the outer 500 μm of the bud had increased, up to *c*. 15 kPa pO_2 immediately within the scale, while the remaining path towards the core remained near levels seen in intact buds at 3 h (**Fig. 3C**). By 72 h, the pO_2 profile of outer 1400 μm of tissue resembled that of the de-scaled buds at 3 h, however the pO_2 of the inner 500 μm had increased,

resulting in a biphasic profile such that the minimum pO_2 along the electrode's transect was *c*. 7 kPa at 1400 μm depth from the scale, while at 2000 μm depth, the pO_2 was >10 kPa (**Fig. 3D**). **Figure 3E** shows the path of the microelectrode in a representative section.

Histological detection of superoxide and hydrogen peroxide

Using replicate buds of the same developmental series and treatment conditions as used for pO_2 microelectrode measurements, we stained for the local accumulation of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), detected as the products of reactions with NBT or DAB respectively. Immediately upon removal from 4 °C (0 h) and after 3 h at 23 °C, $O_2^{\cdot-}$ accumulated in a very confined zone of the meristematic tissue, around the axillary meristems (**Fig. 4A**). After 3 h however, $O_2^{\cdot-}$ accumulation was observed in the cambial meristem tissues. For the first 3 h no H_2O_2 accumulation was detected in tissues around the apical meristem but low levels were observed in the cambial meristem tissue (**Fig. 4E**, **4F**). After 24 h, $O_2^{\cdot-}$ levels were increased in a wider zone of tissues of the apical meristem complex and retained in the cambial meristem tissues, while H_2O_2 was not accumulated in the tissues with the exception of the cambial meristem (**Fig. 4C**, **4G**). At this time point the pO_2 at the core of the bud complex remained low. A more distinct pattern of $O_2^{\cdot-}$ localisation emerged at 72 h, which suggested association with the developing pro-vascular tissues (**Fig. 4D**). At 72 h, no H_2O_2 accumulation was observed in the bud tissues (**Fig. 4H**). By this stage, the pO_2 at the core of the bud complex had increased, suggesting a possible association between the patterns.

To investigate the cell types associated with the distinct $O_2^{\cdot-}$ pattern seen at 72 h, we counter-stained sections to visualise lignin. **Figure 5** shows a clear co-localisation of $O_2^{\cdot-}$ with lignified cellulose as early as 3 h from transfer to 23 °C, but not earlier,

providing further evidence that these are developing pro-vascular tissues. At 0 h, $O_2^{\cdot-}$ accumulation was localised in the meristematic tissues but very little lignin associated with this pattern (**Fig. 5C**, **5D** show magnified images of the boxed areas of **Fig. 5A**, **5B**). By contrast, at 3 h the co-localisation of $O_2^{\cdot-}$ and lignin was observed (**Fig. 5D**, **5E**, **5F** shows the individual and superimposed images). Close inspection of **Figure 5E** reveals the typical ladder-like perforation plates of xylem vessel elements.

DISCUSSION

The experimental system presented here mitigated the potentially confounding effects of endodormancy and the influence of light. Endodormancy in grapevine, as in many perennial trees and vines is primarily overcome by an accumulated exposure to chilling. Adequately chilled buds are termed ecodormant, a qualitative condition that is repressed only by the unfavourable growth environment (i.e. cold) and therefore more comparable to quiescence in other organs and forms of life. Bud burst *per se* does not require the presence of light (Pouget, 1963), although several studies have demonstrated influences of light intensity and photoperiod on organogenesis at other stages of development (Buttrose, 1970, Srinivasan and Mullins, 1981). There is no knowledge of whether photosynthesis may initiate in the bud prior to bud burst. Drawing analogy to seeds, where in several species photosynthesis influences the internal pO_2 even during development or when mature and imbibed prior to germination (Borisjuk and Rolletschek, 2009), we may expect this to be the case in buds. Hence, overcoming endodormancy and excluding light allowed us to accurately and precisely study heterotrophic metabolism during the acute phase of bud burst.

Cells in a quiescent state are defined by very slow metabolic rates, with minimal respiration until environmental or metabolic triggers prime the metabolic systems to resume growth. While several authors have described conserved responses to hypoxia or other oxidative stress across species and life forms (Hochachka, 1986, Jones et al., 2000, Mustroph et al., 2010), it is not possible to construct a generalised description of the metabolic state of quiescent cells or the changes that occur upon the transition to the metabolically active state or subsequent proliferation (Valcourt et al., 2012, Teslaa and Teitell, 2015). The findings of the present study provide new insights into the management of hypoxia when dormancy is broken in quiescent grapevine buds by

exposure to chilling and the subsequent transition to ambient temperatures. While respiration rates are rapidly increased and superoxide accumulation is observed in and around the developing lignified zone of the cambium following the transition to ambient temperatures, the release from the hypoxic state is gradual and occurs in specific regions of the bud as the developmental transition progresses.

A rapid acceleration of respiratory CO_2 production was observed in the buds following the transition from low to ambient temperatures demonstrates the alleviation of the constraints maintaining the quiescent state. This process, which was observed over the 72 h of bud burst measured at 23 °C, resembles the pattern observed during seed imbibition (Bewley, 1997) and in other studies on perennial buds (Hollis and Tepper, 1971, Shulman et al., 1983, Gardea et al., 1994, McPherson et al., 1997, Perez et al., 2008). Measurements of respiratory CO_2 production, do not allow discrimination between TCA cycle activity, fermentation, the pentose phosphate pathway or other pathways. Evidence suggests that fermentation occurs during bud burst under stress conditions and that the imposition of stress accelerates bud burst. For example acetaldehyde and ethanol accumulate in ecodormant grape buds treated with sodium azide, hydrogen cyanamide or heat shock (Ophir et al., 2009). Hydrogen cyanamide, heat shock and hypoxia increase the levels of transcripts that are orthologues of *ALCOHOL DEHYDROGENASE*, *PYRUVATE DECARBOXYLASE* and *SUCROSE SYNTHASE* in ecodormant grapevine buds (Or et al., 2000, Ophir et al., 2009, Vergara et al., 2012b). However in each case, untreated controls showed a slower or weaker transcriptional response with negligible fermentation activities observed during bud burst. These observations suggest that stress-induced changes in transcript profiles do not reflect the transcriptome signatures of developmental regulation of bud burst. Some evidence of pentose phosphate pathway activity was seen throughout seasonal

development in pear buds (Zimmerman and Faust, 1969), and during chilling of potato tubers (Dwelle and Stallkneckt, 1978) or Peony buds (Gai et al., 2013). However, these studies represent quite different physiological states compared to bud burst.

Many plant tissues and organs, including dry seeds, have permeability barriers that reduce oxygen diffusion. In the case of seeds, the hypoxic state may contribute to maintaining quiescence (refer Introduction). The data presented here show that the scales of the dormant bud are a significant barrier to oxygen. Crucially however, the meristematic core of the bud tissues remained in a hypoxic state even when the outer scales were removed. While Iwasaki and Weaver (1977) suggested some acceleration of bud burst in de-scaled ecodormant grapevine buds, removal of the outer scales did not affect the rate of bud burst in our study (data not shown). Schneider (1968) also showed that removal of scales attenuated quiescence of Rhododendron floral buds. However, in these earlier studies there was very limited replication of experiments. Nevertheless, it is conceivable that the buds used in our study were near to 100 % labile and hence very little effect of scale removal would be seen.

The data reported here demonstrate that the pO_2 at the meristematic core of the bud complex was in an hypoxic state for up to 24 h after the environmental trigger to resume growth had caused an increased in respiration. Respiratory CO_2 production rates had increased by 15 % in 24 h and superoxide accumulation was observed in the cambial tissues underlying the meristematic core of the bud complex. By 72 h however, the oxygen profile was biphasic, the oxygen levels within the bud core had increased and superoxide accumulation was pronounced within the pro-vascular tissues. Present data are insufficient to explain the biphasic profile of oxygenation. In the heterotrophic conditions presented, even once the resistance to diffusion of the outer scales and compacted tissues were relaxed, the increased respiratory rates would contribute to

substantial declines in pO_2 with distance into the tissue. Further investigation of the vascular flow and metabolic activities at the core of the bud complex are required. Our group is currently exploring these features, and also the developmental processes and controls that preside in the presence of light, where photosynthesis may contribute to oxygenation even prior to bud burst, as is the case during germination of some seeds (Borisjuk and Rolletschek, 2009).

Vascular development and re-activation of intercellular communication are proposed to be essential early features of the transitions to and from quiescence in plant organs, including grapevine buds (Esau, 1948, Rinne et al., 2001, Paul et al., 2014). Cell expansion, cell wall thickening and the conductivity of plasmodesmata in vascular tissues are all dependent on, or influenced by ROS accumulation (Gapper and Dolan, 2006, Benitez-Alfonso et al., 2011). Ogawa et al. (1997) showed a strong co-localisation of lignin and superoxide (NBT) in vascular tissue of spinach hypocotyls. Moreover, these authors demonstrated that inhibition of CuZn SUPEROXIDE DISMUTASE (CuZnSOD) or NAD(P)H OXIDASE reduced vascular lignin biosynthesis. More recently, ectopic expression of CuZnSOD and/ or ASCORBATE PEROXIDASE (APX) in *Arabidopsis* resulted in enhanced vascular lignin synthesis (Shafi et al., 2015). SOD, APX and catalase were found in cell membranes that had been partially purified from lignin-producing tissues of Norway spruce (Karkonen et al. (2014). Taken together, these data suggest that vascular lignin synthesis is dependent on superoxide and/ or hydrogen peroxide production. It is important to note that hydrogen peroxide did not accumulate in vascular tissues of the buds studied here.

Taken together, the data presented here adds to the growing body of evidence showing that regulation redox and oxygen metabolism are critical to organ development (Considine and Foyer, 2014). The present study demonstrates that during bud burst, the

complex network of enclosed shoot meristems undergoes a controlled transition from hypoxia to increasing pO_2. This transition is accompanied by a highly localised accumulation of ROS in and around the developing cambium and vascular tissues. These data clearly demonstrate the spatial and temporal nature of the control of the oxygen and redox environments within the bud that occurs during the transition from quiescence to burst in heterotrophic grapevine buds.

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FIGURE LEGENDS

Figure 1. Time-series of grapevine bud burst. Single node explants of ecodormant buds were transferred from cool-storage (4 °C) and planted out at 23 °C (dark). Figure shows the progression of bud burst at 0, 1, 3, 7 and 9 days (left to right) at 23 °C. Buds were sampled for the studies presented here at select time points during this development. The figure insert shows a sagittal section of the bud, with the primary (centre arrow), secondary (right arrow) and tertiary (left arrow) bud meristem complexes. When ecodormant (0 days), the bud complex is enclosed by a layer of lignified scales and several layers of bracts. Progressively over 3-5 days we observed expansion of the bud complex and rupture of the outer scales. Within 5-7 days, buds reached the stage of bud burst, according to the modified Eichorn-Lorenz scale (EL4; Coombe, 2004). By 9 days, the first leaves had separated from the shoot apical meristem (EL7). Scale bar main figure = 5 mm, figure insert = 1 mm.

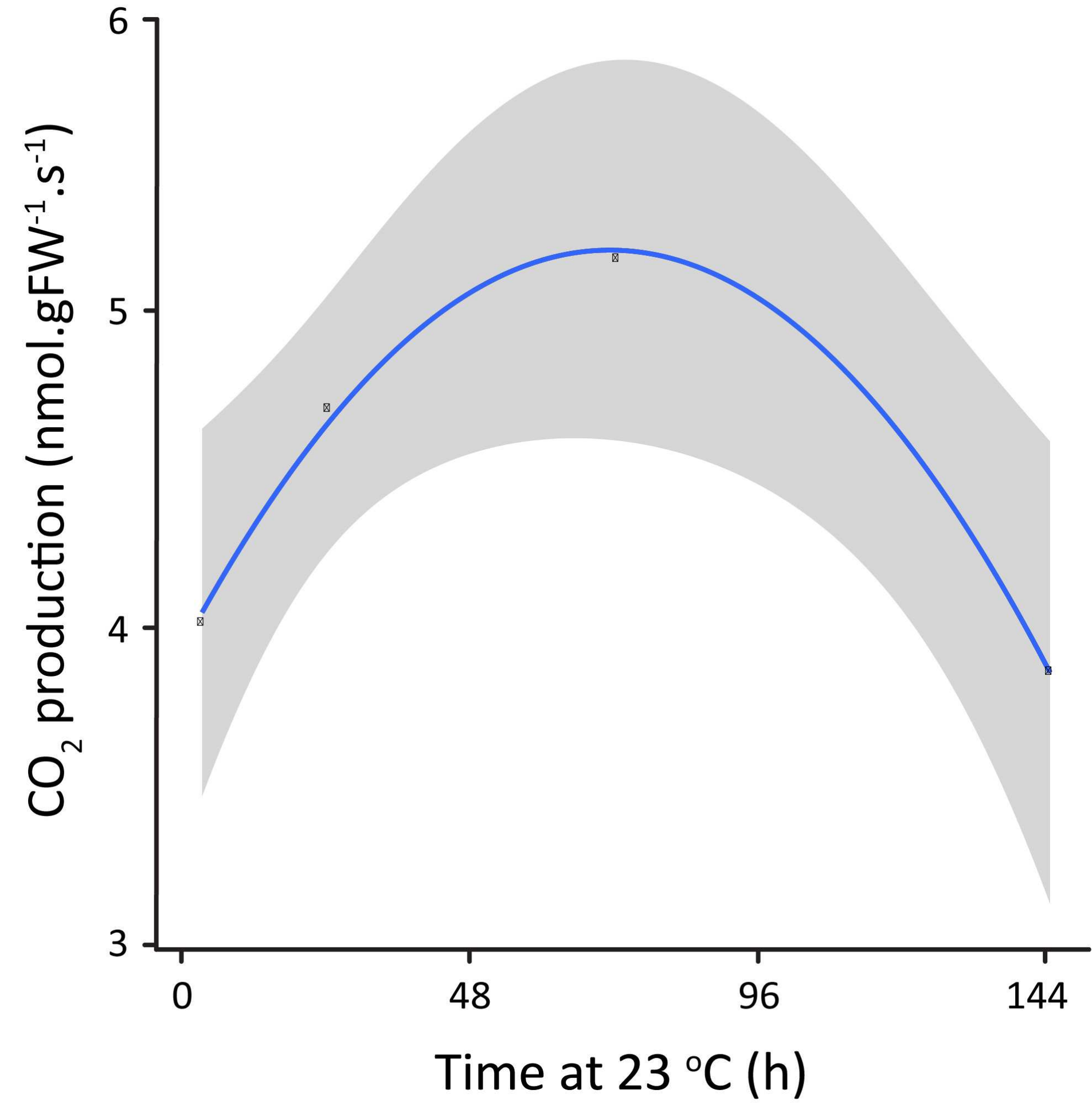
Figure 2. **Respiratory CO_2 production during grapevine bud burst**. Ecodormant buds were transferred from cool-storage (4 °C) and planted out at 23 °C (dark) at zero hours. The rate of CO_2 production was measured on groups of four excised buds with the cut base on agar using an infra-red gas analyser in darkness. Data represent a regression (n = 4 replicates of 4 buds per replicate) +/- 95 % confidence intervals by fitting the time-series of CO_2 evolution to a quadratic equation of the form, $y = \alpha + \beta_1 x + \beta_2 x^2$ (refer Materials and Methods).

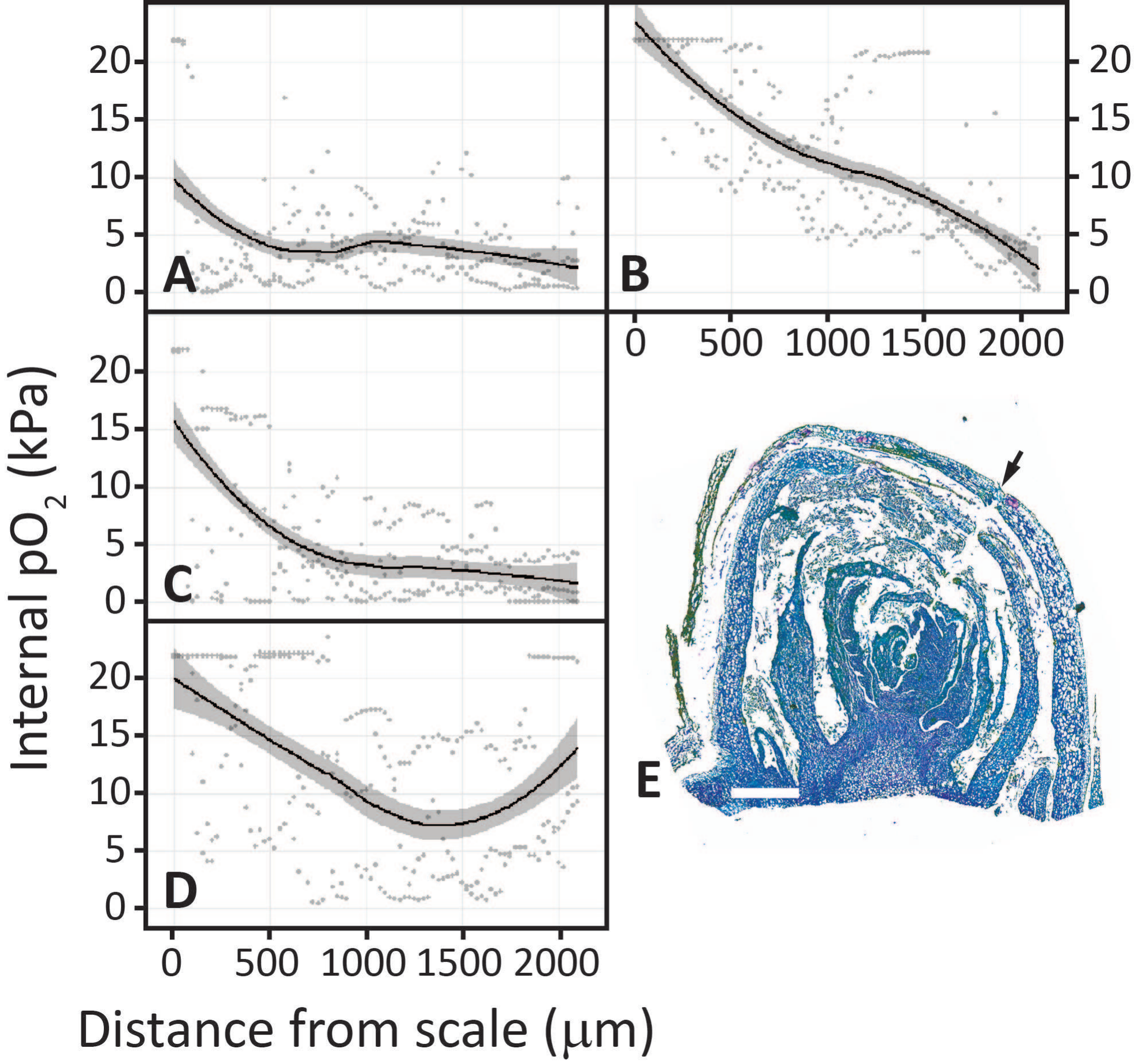
Figure 3. Internal profile of the partial pressure of oxygen (pO_2) during grapevine bud burst. The pO_2 of ecodormant buds, intact (**A** = 3h, **C** = 24h, **D** = 72h) or with the outer scale removed (**B** = 3h) was assayed after time at 23 °C in darkness. Data represent scatterplots of raw data (n = 3), with a regression curve applied and 95 % confidence intervals shown as grey shading. **E** - Sagittal section of the primary bud

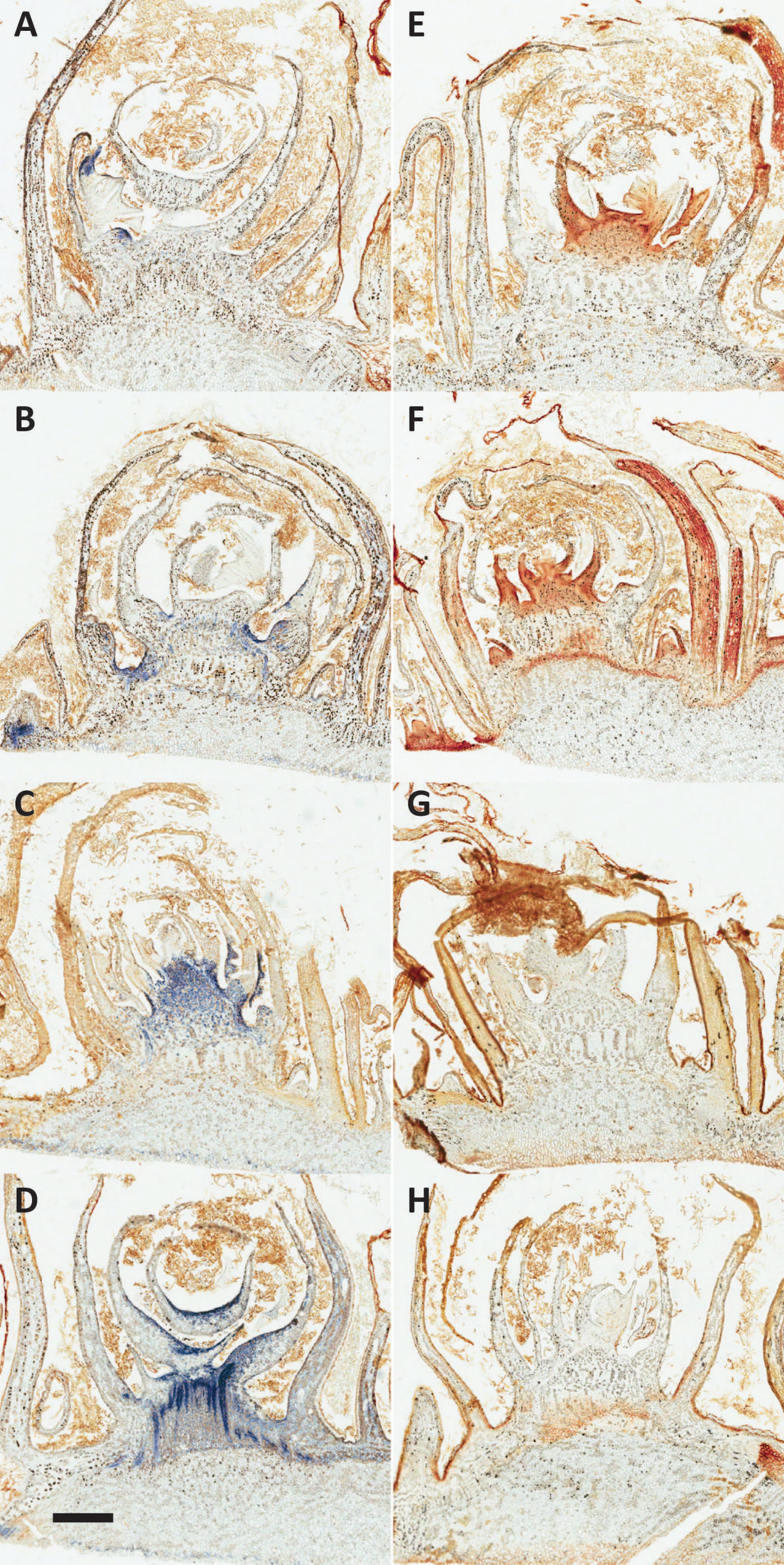
meristem complex, fixed and stained with toluidine blue, showing the path of the O_2 microelectrode from the outer scale (arrow) towards the inner core of the primary bud complex. Scale bar = 500 μm.

Figure 4. Spatial and temporal localisation of reactive oxygen species (ROS) in sagittal sections of the primary bud meristem complex during bud burst. Superoxide (**A**-**D**) and hydrogen peroxide (**E**-**H**) localisation were indicated using nitrobluetetrazolium (NBT) and 3,3'diaminobenzedine (DAB) respectively against fixed sections (20 μm), sampled at 0 h (**A**, **E**), 3 h (**B**, **F**), 24 h (**C**, **G**) or 72 h (**D**, **H**) after transfer to 23 °C. Scale bar = 500 μm. Figures are representative of three independent replicates.

Figure 5. Spatial and temporal localisation of superoxide (A, C, E) as contrasted to lignin (B, D, F) in grapevine buds during the first three hours after transfer to 23 °C. Superoxide (NBT) is localised to latent meristem cells at 0 h, with negligible association with lignified cells (**A**-**D**, indicated by Auramine-O), where **C** and **D** are magnifications of boxed inserts in **A** and **B**. By 3h at 23 °C, superoxide production is evidently associated with lignin, indicative of pro-vascular development (**E-G**), where **G** is **F** superimposed over **E**. Scale bar = 100 μm (**A**, **B**), 20 μm (**C**, **D**), 50 μm (**E**-**G**). Figures are representative of three independent replicates.


6
5
4
3
CO_2 production (nmol.gFW^{-1}.s^{-1})
0
48
96
144
Time at 23 °C (h)


A
B
C
D
E
0
5
10
15
20
0
500
1000
1500
2000
Internal pO2 (kPa)
Distance from scale (μm)


A
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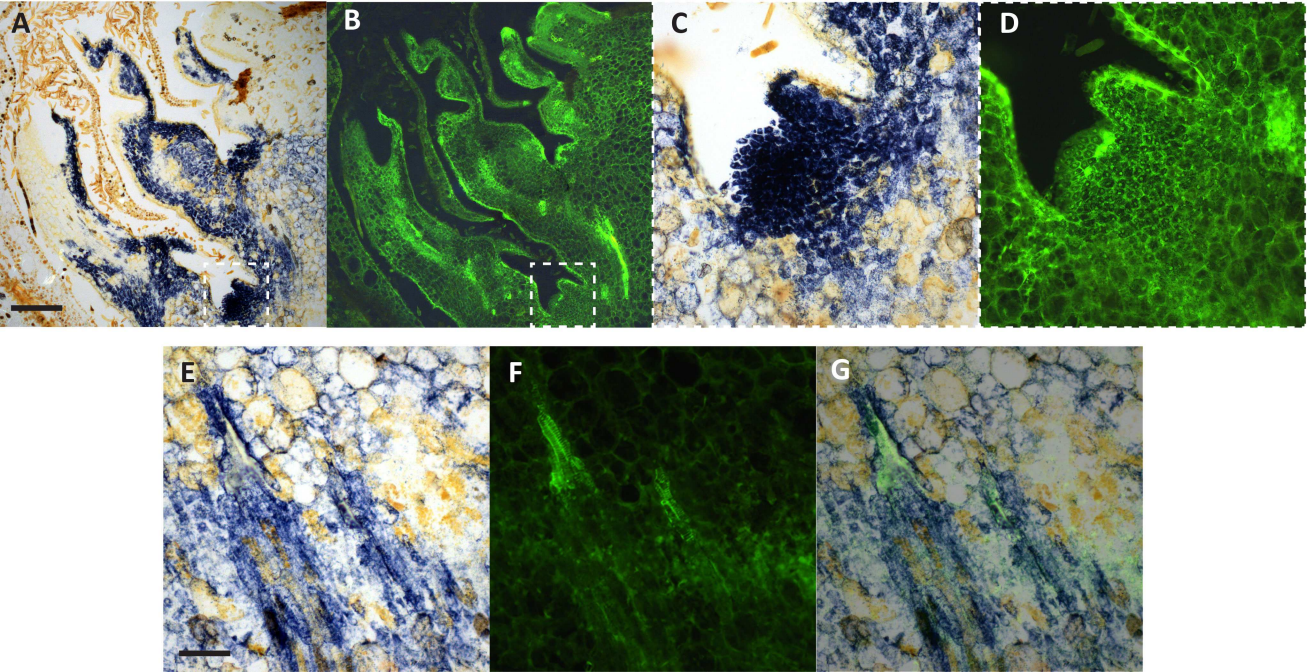
A
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