

Reflexions on some aspects of the interactions among ROS, RNS, and Ca²⁺ in response to a mycorrhizal or a pathogenic fungus

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In vivo redox activities in the apoplast of axenically cultured intact seedling roots (superoxide anion generation, and superoxide dismutase and peroxidase activities) in contact with the compatible arbuscular mycorrhizal fungus (AMF) were clearly attenuated in comparison with those in contact with the pathogenic fungus (PF) or treated with MeJA, even at the early stages of treatment. Contact of roots with the AMF did not enhance the biosynthesis of phenolic compounds (total phenolics, flavonoids, and phenylpropanoid glycosides), while contact with the PF significantly enhanced the biosynthesis of all phenolic fractions. Reactive oxygen and nitrogen species both seemed to be involved in these responses from the first moments of contact, but the fluorescence imaging of roots showed that ROS were mainly accumulated in the apoplast while NO was mainly stored in the cytosol. In conclusion, intact olive seedling roots clearly differentiated between AMF and PF.

Comments

Our study's main interest and originality lies in the plant material used for the redox activities (O₂· generation, SOD, and POX). We worked with intact axenic seedling roots, the whole root of the whole seedling being the source of enzyme in the spectrophotometric measurements. Thus, these redox activities corresponded to the apoplast of roots under in vivo non-invasive conditions, avoiding disruption of the cells or root organization. Hence, in contrast to most of the literature references reviewed measuring activities in crude homogenate extracts that may not

adequately reflect the importance of compartment-specific changes, our results specifically reflect the relevance of the oxidative burst and the antioxidant protective enzymes in the root apoplast (without interference effects from other compartments). Indeed, it is in the apoplast where ROS are first synthesized and required for further defense reactions.¹ Obviously, the techniques for these measurements in our plant material were not easy. They required careful manipulations and numerous controls with the same seedling batch, and repetitions to make our results reliable, as can be seen in the M&M of our referred-to paper and others.^{2,3}

We also must emphasize that the most important results were obtained at very early times (from 5 to 120 min) of the contact between roots and the corresponding fungus (Arbuscular Mycorrhizal Fungus, AMF = *Rhizopagus irregularis*, or Pathogenic Fungus, PF = *Verticillium dahliae*), although the experiments were followed up to 24 h. Also the fluorescence images were obtained from whole intact seedling roots, pre-incubated in the probes for O₂⁻·, H₂O₂, or NO, and then the whole root or the cuts were prepared without fixation or any other treatment. So, these images as well corresponded to in vivo cells and tissues.

The most important results of our experiments can be summarized as follows:

1. From the very early contact times, the oxidative burst (O₂⁻· generation) and the related enzymes (SOD and POX) measured in the apoplast of the intact roots in contact with the PF presented values much greater than those in

control roots and in those in contact with AMF. The PF-treated root values were even greater than those in roots treated with MeJA, the defense reactions phytohormone. Also the phenolic compounds measured in root homogenates showed the same response. Indeed, the defense reactions evoked in roots by AMF were either similar to the control or just a little stronger. We interpreted this as being that the mycorrhizal fungus attenuated the defense reactions of roots in order to facilitate the establishment of the mycorrhiza. This was in contrast to the strong defense response evoked by the pathogenic fungus, PF, described above.

- All the enzymatic activities measured in the apoplast of these *in vivo* roots showed homeostatic oscillations, with SOD and POX roughly in opposition to $O_2^{\cdot-}$ generation, since the excess of $O_2^{\cdot-}$ would induce SOD and POX activities that would keep $O_2^{\cdot-}$ and H_2O_2 accumulation in the apoplast under physiological control. This was so for the controls, and the MeJA and AMF contact treatments, but not for PF contact. In this last case, strong, steady (without peaks or oscillations) $O_2^{\cdot-}$ generation in the root apoplast was observed throughout the time of the experiments, although SOD and POX showed clear oscillations apparently unrelated to the O_2 generation. We thus concluded that PF attack induced a strong oxidative burst that escaped physiological control.
- We also made images of intact whole roots pre-incubated in fluorescent probes for $O_2^{\cdot-}$, H_2O_2 , and NO, and also in transversal cuts, without fixation or any other treatment except washing to remove the excess of probe. Both ROS were strongly generated by the MeJA- or PF-treated roots, but to a much lesser extent by the AMF-treated roots. ROS accumulation, coincident with redox activities, was mainly restricted to the apoplast, at least during the first hours; afterwards (24 h), other cytosolic compartments were also involved in the PF-treated roots. Roots in contact with AMF showed a greater accumulation of NO than the

controls, but much less than that of roots in contact with PF. In contrast with the ROS however, NO always appeared, mainly accumulated in the cytosol, at least during the first hours.

Thus, according to our results, ROS and NO were not produced initially in the same cell compartment, and this must be taken into account in any explanation of the proposed interactions between ROS and NO in the defense reactions, which have still not been fully clarified.⁴

In conclusion, intact olive roots clearly differentiated between mycorrhizal and pathogenic fungi, attenuating defense reactions against AMF so as to facilitate arbuscular mycorrhizal establishment, but strengthening and sustaining defense reactions against PF. Both ROS and NO seemed to be involved in these responses from the first moments of contact, but fluorescence images of the roots revealed that ROS was mainly accumulated in the apoplast (congruent with the redox activities measured in this compartment) while NO was mainly stored in the cytosol.

Hypotheses and Perspectives

There has recently been reported the very important connections between ROS and Ca^{2+} both within and across different cells.⁵ We think it would be very interesting to measure Ca^{2+} oscillations in our roots, as it has been reported that, from the earliest contacts, symbiotic interactions evoke Ca^{2+} oscillations in the cytosol of epidermal root cells, with these oscillations acting as a signal to generate ROS in the oxidative burst and to induce signaling cascades resulting in the biosynthesis of the compounds involved in mycorrhization. A key enzyme has been described as essential to decoding the oscillatory Ca^{2+} signal related to symbiosis in root cells: Ca-CaM-kinase, exclusive of plants, which is only present in roots that can undergo mycorrhization (it has not been found in *Arabidopsis* roots which never form mycorrhizae). These findings could complement our results, perhaps explaining why AMF induced slight oscillations in $O_2^{\cdot-}$ generation while PF induced strong and sustained (without peaks or oscillations) $O_2^{\cdot-}$

generation throughout the time of the experiments. The absence of Ca^{2+} oscillations or Ca-CaM-kinase activity in this latter case would be a good explanation for this effect, and the sustained generation of $O_2^{\cdot-}$ in the apoplast (not under physiological metabolic control) would be one of the causes for the highly deleterious symptoms shown by olive plants infected by this pathogen.

Finally, there is another important point that needs to be clarified in current attempts to understand stress-related ROS signaling: the communication between the ROS produced, on the one hand, in the apoplast by RBOH and other enzymes in the plasma membrane (PM) and cell wall (CW) in the oxidative burst and, on the other, in internal sources such as oxidative organelles (chloroplasts, mitochondria, and peroxisomes). Indeed, there is a great gap in knowledge of the signal communication between these 2 sources of ROS.⁶ Many groups are therefore working with isolated organelles, and have been finding important results concerning the production of ROS and RNS inside them, and then the transmission of these reactive species to the cytosol. But, what comes first? Where is the signal first perceived, and how is it transmitted to then reach the nucleus and induce changes in gene expression? From our perspective, the signals must first be perceived where the corresponding receptors are located. It would then be clear that most biotic and some abiotic stresses, e.g., salinity, drought, heavy metals, or other nutritional stresses, will be sensed at the PM, the location of the respective receptors for biotic attackers and for salinity or drought (cf. the recently discovered drought receptor OSCA1 in *Arabidopsis thaliana*).⁷ In this case, the oxidative burst evoked in the apoplast by RBOH and other enzymes of the PM and CW will be the first event, and then the ROS signal will be transmitted (how?) to the interior of the cell as second messenger to induce signaling cascades (which ones?) including the organelles and ROS (and RNS?) production inside them, and then the release of those species into the cytosol and the nucleus.

But for other abiotic stresses such as heat, cold, excess light intensity, and others for which the identity and exact

location of the receptors are unknown because the whole cell can sense them, the first event might be different, and the most sensitive organelles, such as the chloroplasts, might play the most important part in the initial generation of ROS and RNS. This might also be the case for the defense reactions to those infections in which the pathogen inoculates an avirulence protein (Avr) into the cytosol of the cell, since the receptor (R) in this case is also a cytosolic, not a PM, protein. Further investigations must be conducted to clarify the proposed ROS-NO and Ca²⁺ crosstalk and their respective roles in these responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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