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On the Use of the Own Plant's Defence Compounds to Maintain the Post-Harvest Fruit Quality

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Abstract: This work demonstrates that the own plant defence compounds, like resveratrol in grapes, can be used as a natural and ecological alternative to chemical pesticides. An extract from vine leaves was used to maintain the quality of post-harvested grapes. Specifically, an extract (95% water and 5% ethanol) of such leaves was prepared and, subsequently, different bunches of grapes were immersed in it. The same number of bunches received either water or ethanol (5%) treatment, being both used as blank. Remarkably, on the 14th day after the beginning of the treatment the extract treated grapes, always maintained at room temperature, showed no sign of physical deterioration. In contrast, the two blank samples, i.e.: the water or ethanol treated bunches appeared dehydrated, infected and deteriorated. Furthermore, the external application of the leaf's extract does not modify sensorial, biochemical and nutritional properties of the fruits. The beneficial effects of trans-resveratrol, such as anticancer, antiviral, neuroprotective, antiaging and anti-inflamatory support further the use of this natural compound instead of hazardous synthetic pesticides. Therefore, this study represents a step towards the development of new protocols to maintain the post-harvest fruit quality without resorting to chemical pesticides.

INTRODUCTION

In most climates, fruits can only be grown and harvested during a small part of the year. Yet, consumption of fruit, and hence demand occurs throughout the year. For this reason, large parts of fruit harvests must be stored for extended periods of time before they are sold to the consumers. Obviously, such storage causes considerable losses due to pathogen attack and natural senescence. The traditional solutions to these problems have been storage under controlled or modified atmospheres and the use of synthetic pesticides. Nevertheless, losses exceeding 20% are not uncommon. With respect to the use of controlled or modified atmospheres, though significant advances have been made over the recent years [1,2], no general and efficient methodology is yet available.

Despite the wide literature on sustainability and sustainable agriculture, specially since the publication of the Brundtland Report [3] by the UN World Commission on Environment and Development (WCED), there is still no consensus on the definition of "sustainable agriculture" [4]. Although it seems to be a wide consensus on its importance, in practice, there is no single approach to sustainable agriculture due to its multi-dimensional character, including: economics, environmental and social concerns [5-7]. In this view, during the lasts decades there is a worldwide trend to find new alternatives to control post-harvest pathogenic diseases in a more environmentally and toxicologically safe manner.

The use of chemical pesticides, is nowadays widely accepted as even more dangerous than previously believed [8], and the indiscriminate use of chemicals is generally considered as one of the main undesirable effects of modern agriculture, affecting both human health and wildlife population. The main problems related to the use of chemical pesticides have been identified as: the high frequency of insecticide resistance developed by many insect species, pest resurgence, acute and chronic health problems, environmental pollution and uneconomic crop production [9]. This situation has encouraged the search for alternatives to significantly reduce the use and application of chemical pesticides.

In this work we explore an alternative to the use of both controlled atmospheres and chemical pesticides. Our approach consists of using the plant own pesticides to improve their natural resistance. Natural products of plant's secondary metabolism have been used in "natural" medicine since the early times of human history. An important group of secondary metabolites are the so-called "phytoalexines": antiphatogenic compounds produced by plants after infection or elicitation by biotic or abiotic agents. Under pathogen attack plants evolve sophisticated systems of detection and response to decipher the pathogen signals and induce appropriate defenses. These systems include specific networks that operate through the action of signaling molecules such as salicylate, jasmonate and ethylene and generate the accumulation of pathogen related proteins, phytoalexins or other phenolic compounds [10-12].

The basic function of these chemicals is to protect the plant from attack. Then, a good strategy would first require the identification of the components of the natural defense response in plants. They can be then used both as early and sensitive indicators for spoilage and to enhance resistance.

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For the latter a good strategy will be either to enhance the natural content of these defense compounds or to externally apply them to the harvested fruit to improve its natural resistance. In this way, phytoalexines represent a large reservoir of possible natural pesticides to be used for pest control instead of chemicals [13-16].

A good example of a compound with demonstrated phytoalexinic character is the trans – resveratrol (3, 5, 4'- trihydroxistilbene) which is an antioxidant compound naturally produced by a huge variety of plants as self-defense agent. Its production occurs in vine plants as response to fungic infections and other kind of stresses (UV radiation, chemicals, climatic conditions, etc.) [17-20]. This compound has shown to be fungitoxic at physiological concentrations against Botrytis cinerea [21], which is the most destructive of the post-harvest diseases of table grapes. It also enhances the resistance of vineplants to other pathogens as Plasmopara viticola [22], Phomopsis viticola [23] or Rhizopus stonifer [24]. This rather unspecific antifungal character and its selective accumulation in grape skin [25] makes it a good candidate as a "natural pesticide" against pathogen attack, and therefore, to improve grapes´ natural resistance to fungal infection. Furthermore, trans-resveratrol is known to show important antioxidant properties that could also have positive effects on fruit conservation during storage [26].

Previous work from our laboratory demonstrated the resveratrol elicitation in grapes after infection with Botrytis cinerea [27]. Moreover, the external application of this compound to the fruits considerably improved their natural resistance to spoilage as much as 70 days for apples or 13 days for grapes at room temperature, i.e. without need for storage at cold temperatures [28, 29]. Therefore the starting point of the present work is to collect vine leaves and after dry them to characterize the presence of polyphenols, specifically, trans-resveratrol, which is subsequently extracted for external application to fruits with the aim to improve their natural post-harvest resistance.

MATERIALS AND METHODS

FTIR Analysis

Due to versatility and simplicity, we have employed the Fourier transform Infrared Spectroscopy (FTIR) to analyse and characterize the vine leaves. Nevertheless, a more sophisticated laser analytical method [30] (see below for more details) was used to confirm the presence of the compound, though is availability is not necessary for the protocol here developed, as explained below.

To prepare the sample for FTIR analysis, vine leaves were dried before grinding them to particle size below 25 µm. Afterwards a mixture with KBr was prepared in a weight percentage of 4% and 96% for sample and KBr components, respectively. Once the prepared mixture was homogeneous 100 mg of it was used for each run of transmission or diffuse reflectance measurements.

The spectra were taken by diffuse reflectance with a FTIR Spectrometer (FTIR-8400S from Shimadzu) with a resolution of 4 cm^{-1} and using Happ-Genzel apodization. In all cases, a few scans were enough to observe the broad band over the 3000 - 3300 cm^{-1} region associated to the presence of multiple OH which is of relevance for the present work as it will be discussed later.

Extracts Preparation and Grapes Treatment

The experiments here presented were carried out with an ethanolic extract obtained from Aledo vine leaves. This variety was selected because its high concentration of trans-resveratrol (typically 10 ppm). In order to obtain such extract, the leaves were cut in small pieces and filled in a recipient with ethanol. The quantities used were 8 L of ethanol to extract 3.5 kg of vine leaves. The leaves were maintained in ethanol with no sample agitation and light protected during 7 weeks to ensure the complete extraction of the phenolic compounds. Periodically, a sample was taken and analized by UV-visible and FTIR spectrophotometry to monitor the advance of the polyphenolic compounds extraction.

The treatment was carried out on grapes (*vitis vinifera*, Moscatel variety) directly purchased from the market. In order to minimize effects of different maturity stages between bunches, they were cut in two similar moieties and were incorporated to three different test groups: one group was immersed in water (control), a second group was immersed in a solution of ethanol 5% in water and the third one was immersed in a solution of 5% of the leaves ethanolic extract in water. Each experiment contained 3 half-bunches of approximately the same weigth. The experiments were repeated up to five times to ensure reproducibility of the results.

Laser Desorption and Resonant Ionization Mass Spectrometry

The trans-resveratrol content in the vine leaves was measured using the laser technique developed in our lab which has been widely described elsewhere [25, 30] so only a brief report is given here. It is based on the combination of Laser Desorption (LD) with Laser Resonance-Enhanced Multi-Photon Ionisation (REMPI) coupled to Time-of-Flight Mass Spectrometry (TOFMS) detection. A basic feature of the technique is the absence of any separation method for sample preparation, thus, the combination of Laser Desorption followed by REMPI-TOFMS detection can overcome the main error sources, present in the chromatographic methods generally employed for *trans*-resveratrol analysis in complex samples.

Microbiological Analysis

Weighted mixtures of grapes skin (50%) and vine leaves (50%) were ground and diluted in a sterile saline solution of NaCl 9%. Grape skins and leaves were used in the microbiological work for completeness. However, similar results were obtained by employing grape skins or leaves alone. The polyphenols' content *(pc)* of each mixture was monitored by the intensity of the 3300-3000 cm^{-1} band in the respective FTIR spectrum. Two mixtures called A and B were selected such that their pc_A / pc_B ratio was ca. 4. Furthermore, samples were prepared solving 3 g of each mixture in 30 ml of the NaCl solution, and then successive decimal dilutions were prepared to enable proper colony quantification. 0.1ml aliquots of each dilution were incubated on a Petri dish at 37ºC for 48 hours with potato-dextrose-agar previously poured in it. After this period *colony forming units per gram (cfu/g)* were counted following standard procedures [31]. This protocol was repeated three times for each A and B sample.

RESULTS AND DISCUSSION

Fig. (**1**) shows the Fourier Transform infrared Spectrum of different samples: namely, pure stilbene (top panel), pure trans-resveratrol (bottom panel) and vine leaf powder. The same percentage of sample (4%) and KBr (96%) was used for each analysis. Due to the presence of the resveratrol in the dried vine leaf, its spectral band shows more similarity to that of pure resveratrol than to that of pure stilbene. To further confirm the presence of the resveratrol in the dried vine leaf, we have applied our laser analytical technique to the samples; Fig. (**2**) shows the mass spectrum obtained with this technique; the peaks corresponding to stilbene (added as internal reference), resveratrol and quercitrin can be noticed. As it is well known, the = C – H stretching in compounds of the form R_1 – CH = CH – R_2 (cis or trans) and R_1 R_2 C = CH – R_3 show vibrational bands in the 3040 – 3010 cm^{-1} region. Furthermore, if the C – H stretching is originated in an aromatic compound it will occur in the 3080 – 3030 cm^{-1} band.

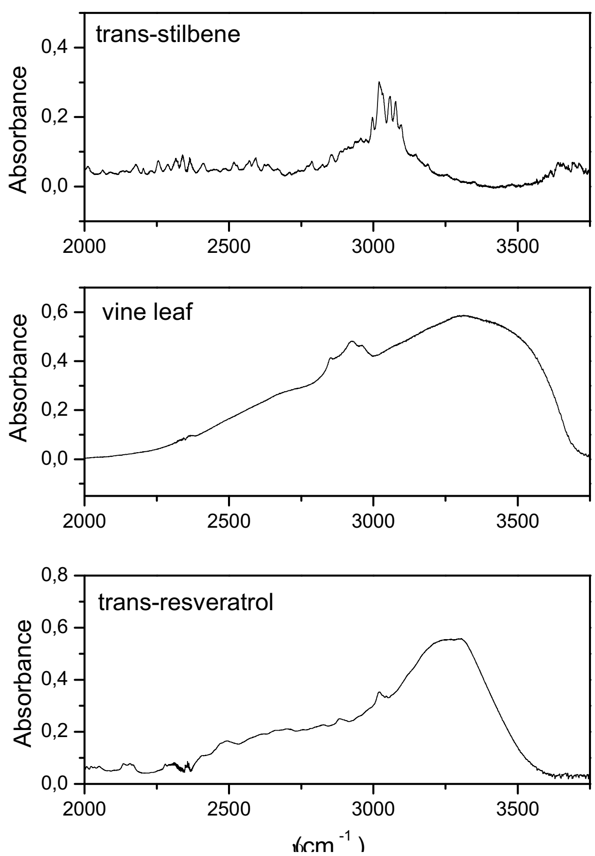


Fig. (1). Fourier Transform infrared Spectrum of different samples. Top: Pure trans-stilbene; Center: vine leaf powder; Bottom: pure trans-resveratrol. The spectrum is the average of 50 scans.

Comparing the 3500 – 2500 cm^{-1} spectral region of both trans-stilbene and trans-resveratrol (Fig. **1** top and bottom, respectively) it becomes clear how the trans-resveratrol band is wider than that of trans-stilbene. For example, whereas the absorption intensity decreases sharply toward the blue region after 3000 cm^{-1} in the trans-stilbene, the absorption intensity

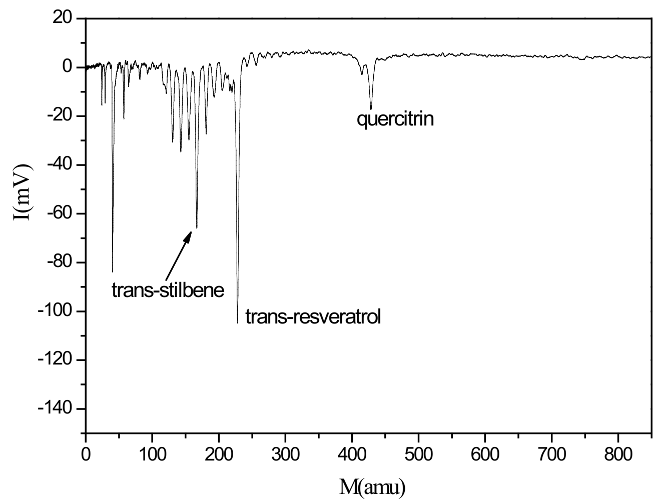
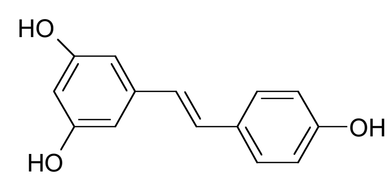


Fig. (2). Mass spectrum obtained from a sample of vine leave by laser desorption coupled with resonant ionisation spectrometry. The resveratrol peak is indicated. See reference 28 for experimental details.

decreases more gently in the trans-resveratrol reaching even the 3500-3400 cm^{-1} zone with non-negligible values. This feature is clearly manifested by the shift in the maximum band value. These values correspond to 3000 cm^{-1} and 3350 cm^{-1} for the trans-stilbene and trans-resveratrol, respectively. Since the basic difference between both compounds is the presence of three OH groups in the resveratrol, as can be seen in their structural formula displayed in Fig. (**3**), we conclude that the wider band and shift in the maximum value are due to the presence of such associated OH.

(**A**)



(**B**)

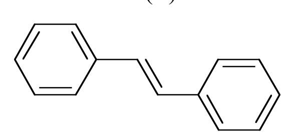


Fig. (3). Structural formula: (**A**) trans-resveratrol; (**B**) trans-stilbene.

Of relevance for the matter under consideration are the expected values of the OH stretching depending on the OH type of interaction as listed in Table **I** [32, 33]. After inspection of these values, it is evident the observed spectral band of the trans-resveratrol is more consistent with the strong and wide 3400 -3230 cm^{-1} band due to intermolecular OH interaction arising from a non-free i.e. associated OH, most likely in the form of a polymer as one would expect in solid trans-resveratrol.

It is very interesting to notice how this wide band featuring the presence of non-free OH is also observed in the vine leaf spectrum suggesting the persistence of such associated OH, present in polyphenols, even in the dried leaves, and therefore making possible its extraction and subsequent application as described in the present investigation.

Table I. OH – Stretching Frequencies in cm^{-1} [Adapted from Ref. 32]

OH - Type		band	Shape	Intensity
Free		3670-3580	sharp	variable
Associated	Intermolecular Dimer	3550-3450	sharp	variable
	Intermolecular Polymer	3400-3220	wide	strong
	Intramolecular	3590-3420	sharp	variable
	Coordination Complex / Chelates	3200-1700	wide	weak

As a result, an ethalonic extract of such leaves was prepared and, subsequently, different bunches of grapes were immersed in this ethanolic extract (95 % water) while the same number of bunches received either water or ethanol 5% treatment. A picture of the three samples is shown in Fig. (**4**) corresponding to the 14th day after the beginning of the treatment. It is remarkable to observe how the extract treated grapes maintain a physical aspect with no sign of losses or deterioration. On the other hand, the water or ethanol treated bunches, appear dehydrated, infected and deteriorated. This experiment was repeated up to five times with similar results.

Sensory and biochemical analysis of these samples were implemented following a standard procedure already described elsewhere [28]. The results indicate that the external application of this extract, from the own plant's leaf, does not modify sensorial, biochemical and nutritional properties of the treated fruits compared with those of non-treated, ones.

To demonstrate the improved natural resistance of the post-harvest grapes is due to the antifungic and antimicrobiological character of polyphenols content present in the vine leaves a microbiological test was carry out. Following the microbiological analysis protocol described in Material and Section Methods and using the same quantity of both A and B samples, the respective colony forming units per gram were counted after sample incubation in potato-dextrose-agar. The resulted *cfu / g* are displayed in Fig. (**5**).

As an example to illustrate the difference observed from the A and B samples the picture enclosed in Fig. (**6**) shows the significant difference against the microbiological infection observed in an individual essay. Looking at both Figs. (**5**,**6**), it is evident how the sample with lower polyphenol-resveratrol content developed a higher number of *cfu / g*.

SUMMARY AND CONCLUSIONS

The main conclusion of the present work is the significant improvement of the post-harvest resistance of grapes when these fruits are immersed in a solution formed by a 95% water and 5% ethanol vine leaves' extract. Thus, it was remarkable to observe that while at the 14th day of treatment the extract - treated grapes maintained a physical aspect with no sign of deterioration, the two other samples, i.e.: the water or ethanol treated bunches, appeared dehydrated, infected and deteriorated.

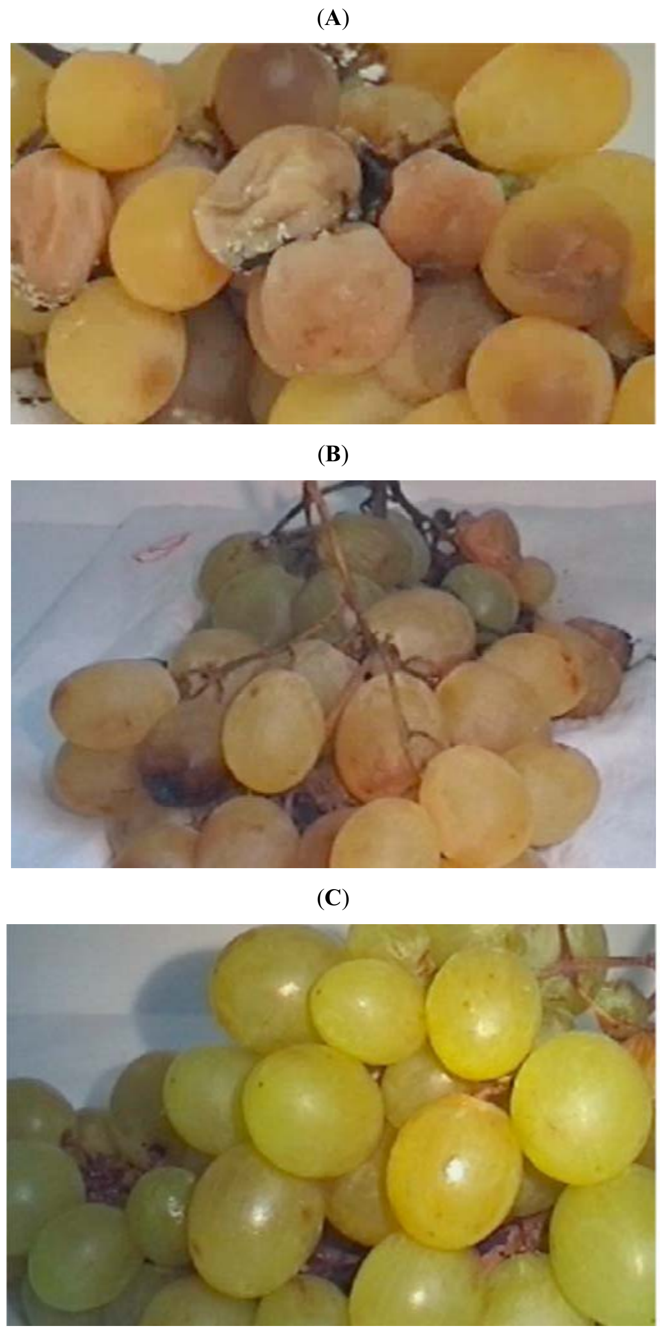


Fig. (4). Grapes stored at room temperature during 14 days after different treatments: (**A**) water; (**B**) ethanol 5% and water 95 %; (C) leaves extract 5% and water 95 %.

The observed positive influence of the natural extracts has been attributed to the presence of polyphenols as monitored by their IR absorption band and demonstrated by specific microbiological analysis. Indeed, analysis performed with these natural samples clearly demonstrated how the higher the content of polyphenols the lower the number of colony forming units after proper cultivation in adequate growing media.

Hence, the results reported in the present investigation may represent a step forward in the use of natural and

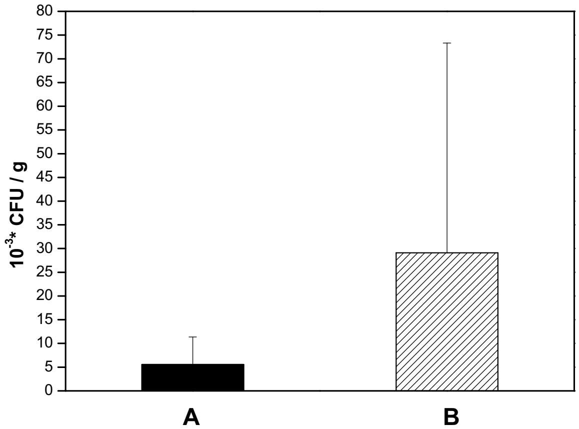


Fig. (5). Average colony forming units per gram for sample A and B after incubation in potato-dextrosa-agar at 37ºC for 48 h. Sample A contains 4 times more polyphenols than sample B. See text for further details

(A)

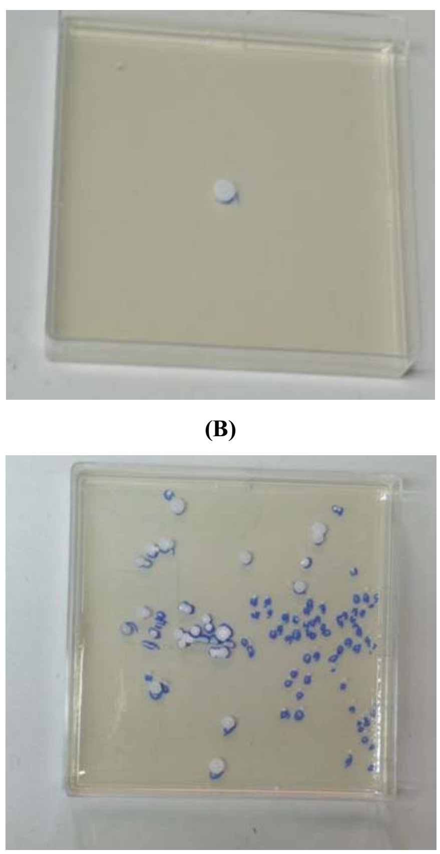


Fig. (6). Typical picture of individual Petri dishes after incubation in potato-dextrose-agar at 37ºC for 48 h. Notice how sample shows no presence of colony forming units whereas almost a dozen of them are manifested in sample B. See text for comments.

ecological methods to improve the post-harvest resistance of fruit paving the way to device an alternative to the use of chemical pesticides. Furthermore, this new approach could be of great importance in Agriculture, especially for countries that cannot afford the high cost of chemical pesticides. Interesting aspects of the new methodology are: (i) the added value of the vine leaf which currently is an agriculture sub-product of little, if any, value and (ii): the low cost of the applied protocol. Indeed a simple (low resolution) FTIR is the only equipment required to select the most suitable vine leaves from which to extract the 'natural pesticides'. In addition, the low cost of the leaves as an agriculture sub-product, facilitates the possibility to implement a commercial use of the protocol developed here. The beneficial effects of trans-resveratrol, such as anticancer, antiviral, neuroprotective, antiaging and anti-inflamatory support further the use of this natural compound instead of hazardous synthetic pesticides. Work is now in progress to apply the ethanolic extract in the form of spray with the objective to develop a more commercial treatment.

ACKNOWLEDGEMENTS

This research received financial support from the Ministerio de Ciencia y Tecnología of Spain (grant CTQ2007-61749). Vine leaves were provided by C.R.D.O. Vinalopó (Alicante, Spain).

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Received: March 12, 2008 Revised: April 24, 2008 Accepted: May 8, 2008

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