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Sodium arsenite effect on *Vitis vinifera* L. physiology.

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Abstract

Sodium arsenite (NaAsO_2) was especially used as a dormant spray to control grapevine trunk diseases (GTDs) in European vineyards until 2003 when it was banned. It was an efficient product but it was banned due to high risk for human health and the environment. Now, as one of the consequences with climatic changes, GTDs threaten the sustainability of vineyards since no similar and efficacious sprays are presently available to reduce the impact of GTDs. Research efforts were devoted to identify other active ingredients and biological control agents but they remained limited in term of efficacy. New solutions might follow from a better understanding of the modes of action of sodium arsenite which are currently lacking, specially its impact on grapevine physiology. For this study, grafted plants cv. Tempranillo were sprayed by sodium arsenite at the end of the winter. During the vegetative period, the impact on plant physiology was studied by measurement of the photosynthetic activity, the vine growth and development, and some defense responses. Our results showed that arsenic was translocated throughout the vine with an increasing gradient from the leaves to the root system, that photosynthesis was firstly reduced and then stimulated, and that plant tolerance responses were induced especially antioxidant system. The activation of grapevine defense responses by sodium arsenite could be a complementary action to fight fungal pathogens in addition to the fungicide effect.

Keywords: sodium arsenite, Tempranillo, photosynthesis, plant physiology, plant response, translocation

1. Introduction

In agriculture, arsenic-based compounds were used firstly as insecticides in the 18th century. Such treatments were applied to control *Leptinotarsa decemlineata* in potatoes (Riley, 1876), *Aclypea opaca* in sugar beet (Grosjean, 1896) and *Anthonomus grandis* in cotton (Robinson, 1926) and *Anthonomus quadrigibus* in apple tree (Quaintance and Scott, 1912) as examples. In the 19th century, arsenic compounds were then used as herbicides in either their inorganic forms, like sodium arsenite, or their organic forms (Schultz and Thompson, 1925; Hood, 1985). In viticulture, they were also employed as insecticides against *Sparganothis pilleriana* (Marchal, 1919), *Eupoecilia ambiguella* (Capus and Feytaud, 1909) and *Lobesia botrana* (Capus and Feytaud, 1909). More recently, their use as fungicides was tested at the beginning of the 20th century, especially to manage grapevine trunk diseases (GTDs) (Ravaz, 1919, 1924; Viala, 1926, Desaché et al., 1995), anthracnose (Vergnes, 1957) and Phomopsis cane and leaf spot (Hewitt, 1951). Other sporadic applications to limit the attacks by snails was reported by Vidal (1947).

Due to the high risks of sodium arsenite for humans and the environment (Spinosi et al., 2009), its use in agriculture was banned in Europe, especially in 2003 for viticulture. In viticulture, this withdrawal was not without consequences since no other active chemical spray treatment was available to reduce the impact of GTDs. These diseases are due to a complex of fungal pathogens which **are inhabit wood** (Mondello et al., 2018a). For GTDs, fungi infect the vine especially by pruning wounds which then colonize the trunk and other woody organs (Mugnai et al., 1999; Bertsch et al., 2013). The pathogens may live inside the vine as asymptomatic endophytes and after some period of latency that is typically interrupted by stress to the vine, the vine might express foliar symptoms and be thus considered as a symptomatic vine. **GTD foliar symptoms can be characterized by neither a sudden wilting of the whole plant or part of the plant, namely apoplectic symptoms or apoplexy, or discoloration with a typical tiger-stripe pattern or chlorosis in the case of the chronic form (Mugnai et al., 1999; Mondello et al., 2018a).**

A recent International Organisation of Vine and Wine (OIV) publication reported that in European countries such as France, Spain and Italy, the incidence of GTDs varied from 8 to 19% (Fontaine et al., 2016) and 13% of unproductive vineyard represented a loss estimated of €1 billion in 2014. In USA, especially California, and in Australia, losses were US \$ 260 million and AUS \$8.3 billion per year. Until 2003 and especially in Europe, the sodium arsenite was the sole product available to inhibit foliar symptoms of GTDs, especially Esca diseases. The effect was less against *Eutypa lata*, causal agent of eutypa dieback, and on

Botryosphaeriaceae spp. close to this reported on esca pathogens (Larignon et al., 2008). Since its ban, their incidence has increased because no product as efficient as sodium arsenite was available for application. Researches were conducted to control Esca disease and they were based especially on pruning wound protection (for review Mondello et al., 2018a), the main way for the causal agents to infect the grapevine, by trunk renewal (Mondello et al., 2018b), and by chemicals or biocontrol agents, prophylactic methods or the use of biostimulant/elicitors (for review Mondello et al., 2018a,b).

The limits to develop a new product with similar effects induced by sodium arsenite to control Esca disease are the gaps of knowledge on its mode of action. To date, no definitive mechanism has clearly been identified to explain how sodium arsenite acts in suppressing foliar symptoms. An explanation could be the effect of arsenic on plant physiology whose resulting perturbations could induce the appearance of symptoms (Magnin-Robert et al., 2011, Magnin-Robert et al., 2017, for review Fontaine et al., 2015). On this basis, it seems relevant to take an interest in the effects of sodium arsenite on the grapevine physiology. Nevertheless, no scientific publication deals specifically with the incidence of arsenic compounds on grapevine physiology. After the vineyard treatment with sodium arsenite, Carbonell-Barrachina et al. (1997a) observed a decrease of sodium arsenite concentration throughout the vegetative cycle and no accumulation of arsenic in leaves and fruits after three-years of treatment, due to a possible exudation into the soil from roots. Similarly, Larignon et al. (2008) conducted sodium arsenite treatment in vineyard and measured a greater concentration of arsenic in the trunk than in the leaves and the fruits, especially in the wood necrosis areas. They also reported no change in the contents of proanthocyanidic tannins and total soluble phenolic compounds in the healthy wood and in the periphery of the white rot. Finally, Santos et al. (2006) noticed that *in vitro* grapevine plants, treated by sodium arsenite, developed leaf senescence symptoms like a decrease of chlorophyll content, of leaf fluorescence and of growth in fresh weight, as well as an increase of lipid peroxidation. For other crops, few papers reported that this compound can penetrate into woody tissues and move inside the plant from roots to shoots and berries, and can be excreted in the soil (for review Li et al., 2016, Latowski et al., 2018). Regarding its impact on plant physiology, numerous disorders are triggered by arsenic, leading to plant growth and productivity decline, the alteration of ATP synthesis and photosynthesis, and the impact on membrane integrity and nutrient acquisition (for reviews, see Abbas et al., 2018, Chandrakar, 2018, Kofroňova et al., 2018). Due to the interactions of As(III) with sulfhydryl groups (-SH) of enzymes and the

ROS generation, plant metabolism is severely altered, notably the carbohydrate, lipid and protein metabolisms, and the DNA structure (for review Finnegan and Chen, 2012, Abbas et al., 2018). Detoxification mechanisms were also induced in plants but not studied intensively (Srivastava et al., 2007; Kumar et al., 2015).

There was interest to develop a mimic product to sodium arsenite to optimize the control of GTDs, especially Esca disease but also both Eutypa and Botryosphaeria diebacks, and other diseases managed in the past by the use of sodium arsenite. This involved the understanding of the relationship between the application, its penetration and translocation within the plant, and the impact on growth and physiology all may help to find a mimic product. The main goal of this study is thus to intensively study the impact of sodium arsenite on grapevine growth distinguishing both aerial and root systems, and on physiology focusing on the carbohydrate metabolism and plant defense response. For this reason, photosynthetic activity was measured and the expressions of targeted genes especially involved in defense response and stress tolerance were monitored.

2. Material and Methods

2.1. Plant material, treatment with sodium arsenite and sampling

In 2013, 1-year-old *Vitis vinifera* cv. Aragonez (= "Tempranillo"), defined as susceptible cultivar to GTDs, grafted on rootstock 1103P grapevine were potted individually in a 1 liter pot containing a sandy soil mixture (one-third sand, one-third soil, and one-third organic matter) and they grew under natural conditions (Figures 1S, 2S). In 2015 (March 5th) and in 2016 (March 10th), dormant vines were sprayed with 1250 g/hl of sodium arsenite (Pyrasca), i.e. the vineyard concentration normally applied. The solution was sprayed using a manual sprayer until runoff. The experimentation was conducted twice and according to a completely randomized design: in 2015, 10 control plants and 10 plants treated with sodium arsenite according to the vineyard concentration (1250 g/hl); and in 2016, 20 control plants, 20 treated plants at 1250 g/hl and 20 treated plants at 312.5 g/hl of sodium arsenite, i.e. one quarter of the prior application rate.

Samples were collected at flowering (half of the plants) and at berry maturity (half of the plants), respectively approximately 3 months (T+90 days in 2015 and T+87 in 2016) and 6 months (T+183 days in 2015 and T+178 days in 2016) after the treatment date (T). The collected plants were used to measure morphological parameters and quantify total arsenic concentration. Then, samples of leaves in the intermediate zone, green stems and root for each treatment were frozen in liquid nitrogen and then stored at -80°C until molecular analysis.

Flowering and berry maturity were selected since they are 2 significant phenological stages in the grapevine reproductive development and because of important sap flows that could promote the translocation of arsenic within the whole plant.

2.2. Quantification of total arsenic

Total arsenic was quantified at flowering and at berry maturity, i.e. 3 and 6 months after the treatment respectively, in the grafted plant and soil. For the plant, the analysis was performed on leaves, on the trunk below the graft/bud union and on roots. Ten (5 controls and 5 treated) and 15 (5 controls, 5 As treated, 5 As $\frac{1}{4}$ treated) samples per treatment were analyzed in 2015 and 2016 respectively. Each plant sample was cut in small pieces of 1 cm³. Plant and soil samples were then lyophilized.

All grapevine organs (roots, trunk and leaves) and soil (0.25 g) samples were digested with 3.0 mL of 69% nitric acid (trace analytical grade, Acros organic) and 1.0 mL of ultrapure water for 35 min at 200°C after 10 min of temperature increase in a microwave digester (Anton Paar, ECO). After mineralization, the volume is made up to 20 mL with ultrapure water. Blanks were prepared in the same way, omitting the sample. The obtained digested solutions are 20 times diluted and the concentrations of total arsenic in the grapevine and soil samples were determined using inductively coupled plasma-mass spectrometry (ICP-MS). ICP-MS (Agilent 7500ce) is equipped with a Peltier cooled Scott spray chamber, a pneumatic nebulizer (1 mL min⁻¹, Glass Expansion), and an inert PFA sample introduction system. The 7500ce instrument uses a collision reaction cell with Helium gas technology to remove polyatomic interferences (mainly ArCl⁺). The optimized operating conditions are summarized in Table 1S. The monitored isotope was ⁷⁵As and ¹²¹Sb at 10ppb was used as internal standard (prepared from 1000 mg L⁻¹ As and Sb certified standard solutions (CPI International) diluted in 1% v/v HNO₃ solution). All the grapevine samples were analyzed in triplicates.

2.3. Measurement of plant growth

On each plant, the fresh weight and the volume of the root, and the number, the length and the diameter of the shoots were measured according to the laboratory protocol in order to evaluate the effect of the treatment on the plant development. The root system volume was estimated by water displacement. To avoid bias linked to the smaller shoots lastly grown, the mean diameters and lengths of the shoots corresponds to the means of the first three thicker and longer shoots. The shoots diameter corresponds to the diameter at the base of the first internode. The measurements were realized at both kinetic points, flowering and berry

maturity, and each year. Five biological replicates per condition and per kinetic point were used in 2015 and ten in 2016.

2.4. Measurement of photosynthetic activity

Net photosynthesis (A_n), stomatal conductance (g_s), internal leaf CO_2 concentration (C_i) and transpiration (E) were determined simultaneously on leaves with a portable infrared gas analysis system (Li-Cor Model 6400-XT, Lincoln, NE) using a standard leaf. Water use efficiency (WUE) was determined by the ratio of (A_n/g_s) which provides the loss of water relative to the CO_2 assimilation (Epron and Dreyer, 1993). Measurements were conducted between 8:00 a.m. and 12:00 a.m. before the “midday depression” (i.e., the minimal A_n linked mainly to stomata closure) (Chaumont et al., 1994). The infrared gas analysis system was equipped with a clamp-on leaf cuvette that exposed 6 cm^2 of leaf area. Environmental conditions during photosynthetic measurements were the following: photosynthetic active radiation = $1,000\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$, relative humidity $\approx 50\%$, and chamber temperature of 24°C . CO_2 was maintained at a constant level ($380\text{ }\mu\text{mol.L}^{-1}$) using a LI-6400-01 CO_2 injector with a high-pressure liquid CO_2 cartridge source. At the same time and only in 2015, the photosystem II activity ($\Phi PSII$) was evaluated using a FMS2 chlorophyll fluorometer (Hansatech Instruments, United Kingdom). The photosynthesis measurements of each plant correspond to the mean of 3 measures made on 3 leaves located at the bottom, middle and top of the canopy. They were conducted on 5 biological replicates per condition in 2015 and on 10 biological replicates per condition in 2016. Measurements were performed on the same leaves from flowering (i.e. 3 months after treatment) until the berry maturity (i.e. 6 months after treatment) and every 2 weeks.

2.5. Molecular analysis by qRT-PCR

The analysis was performed on leaves, green stems and roots of control and treated plants collected at timepoints flowering and berry maturity, as for other analyses. For each plant, after **grinding** of the different samples, total RNA of leaves, green stems and roots were extracted from 50 mg, 100 mg and 150 mg of powder respectively. Total RNA was isolated using a β -mercaptoethanol and sodium acid-based protocol according to Spagnolo et al. (2012) for green stem and Magnin-Robert et al. (2014) for leaves and roots. In total 150 ng of total RNA was reverse-transcribed using Verso SYBR 2-step QRT ROX enzyme (ABgene, Surrey, UK) according to the manufacturer’s protocol. PCR conditions were those described by Bézier et al. (2002). The expression of selected genes according to the organ, summarized in

Table 1, was tracked by quantitative RT-PCR. The data were analyzed using CFX Manager software, and the relative levels of gene expression were determined following the method of Hellemans et al. (2007) with either EF1 α (leaf, green stem and root), 60SRP (leaf) or UBE2 (green stem and root) used as internal reference genes according to the organ (**Table 1**). Results correspond to the relative gene expression in organs of treated plants at flowering and at berry maturity compared to the controls at corresponding timepoints. The value obtained for one treated plant correspond to the mean of the values of the relative gene expression of the treated plant compared to each individual control. In 2015, five biological replicates were used per condition and ten in 2016. The analyzed genes were considered up- or down-regulated when the value of relative expression was >2-fold or <0.5-fold compared to the controls, respectively.

2.6. Statistical analysis

Statistical tests were performed on R 3.3.3 software. For the arsenic content analysis, a Shapiro-Wilk test was done to check the normality of data. As the normality was not assumed in all cases, a nonparametric test of bivariate Wilcoxon was performed when the mean of arsenic and control were compared. The meaning of different symbols is ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). While the mean of three modalities were compared (As, As $\frac{1}{4}$ and **Control**), a Kruskal-Wallis non-parametric test were led and significant differences between groups were indicated with letter (when letters are different, groups are significantly different with a p value < 0.05).

For the morphological and photosynthetic parameters, the statistical differences between the value of the treated plants, either with the full concentration of sodium arsenite or one quarter of it, and the one of the control plants were analyzed. According to the normality of the data and the equality of the variances, the comparisons of the means two by two were performed by Student's t-test if both normality and equality of variances were assumed, Aspin-Welch 's test if there was no equality of variances and Mann-Whitney's test if the normality was not assumed. Statistical tests were realized using R 3.3.3 software. Significant differences between each treated condition and the corresponding controls are marked by *at $p < 0.05$, ** at $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1. Concentration in total arsenic on grafted-plants

Similar trends are reported in 2015 and 2016 (Figure 1, Table 2) for all the studied organs, i.e. leaves, trunk and roots, and in the soil. Globally, the concentration of total arsenic was significantly higher in treated grafted-plant than in controls (Figure 1). We noted that naturally a small quantity of arsenic was detected in all the plant organs of the controls for both sampling times, less than 1 mg/kg of DW (less than 3 mg/kg of DW in the soil). From flowering to berry maturity, we observed a significant increase of total arsenic content especially in leaves ($p < 0.001$) in 2015 and 2016. The average concentrations of arsenic increased from the leaves to the root system, with close to 10 mg/kg DW in leaves and 18 mg/kg DW in roots at timepoint 6 months. In the soil, the average concentration of arsenic was higher than inside the plant and could reach 50 mg/kg DW 6 months after treatment. Similar trend was noted for the plants treated with $\frac{1}{4}$ rate of sodium arsenite, and the concentrations were intermediate between control and full sodium arsenite treated plants.

3.2. Growth of grafted plants

Control and treated plants expressed neither GTD foliar symptoms, nor of any other pests or diseases. In 2015, the treatment with sodium arsenite significantly affected the growth of the plants by decreasing the number, the length and the diameter of the green shoots especially at berry maturity (Table 3). Both the fresh weight and the volume of the root system tend to be lower for treated plants than for controls (Table 3). Similar trends were observed for plants sampled either at flowering or at berry maturity. In 2016, the treated plants showed also a global reduction of their growth but these differences were not significant at both flowering and berry maturity time (Table 2S). No specific alterations were observed on the growth of plants treated with $\frac{1}{4}$ rate of sodium arsenite.

3.3. Photosynthesis measurements

Gas exchange data for year 2015 are shown in Figure 2 and presented according to the number of days after the treatment date (T). A_n was higher in control than treated plants from flowering, T+67 days ($7.9 \mu\text{mol CO}_2\text{m}^{-2}\text{s}^{-1}$ in controls and $5 \mu\text{mol CO}_2\text{m}^{-2}\text{s}^{-1}$ in treated) to bunch closure, T+119 days ($7.8 \mu\text{mol CO}_2\text{m}^{-2}\text{s}^{-1}$ in controls and $3.4 \mu\text{mol CO}_2\text{m}^{-2}\text{s}^{-1}$ in treated), and then, A_n was higher in treated plants than in control until berry ripening T+167 days ($5.6 \mu\text{mol CO}_2\text{m}^{-2}\text{s}^{-1}$ in controls and $8.7 \mu\text{mol CO}_2\text{m}^{-2}\text{s}^{-1}$ in treated). Similar trend was observed for both g_s and E . C_i was either similar (at timepoints T+67, T+74, T+104, T+119 days) or higher (at timepoints T+92, T+133, T+167 days) in treated plants compared to controls. For ϕPSII , the activity was lower in treated plants than in controls until bunch

closure (T+133 days) and then, similar to the controls to reach 0.6 at timepoint T+167 days. Compared to the experiment in 2015, similar trends were observed in 2016 but the differences were less huge the for full treated plants compared to the controls (Table 3S). For the grafted plants treated by ¼ sodium arsenite concentration, no significant differences during the measurement series were reported compared to the controls except for A_n , C_i and WUE at T+130 days (Table 3S). ϕ PSII was not measured in 2016 since no relevant changes were noted in 2015.

3.4. Molecular analysis

In 2015 and at flowering, most of changes in treated plants consisted of the upregulation of *SOD* in leaves, *CHV5* and *STS* in green stems and *GST1*, *HSP α* , *CHV5* and *STS* in the root system (Table 4). In the green shoot, *TIP1* encoding for an aquaporin was down-regulated. At berry maturity, i.e. 6 months after the treatment with sodium arsenite, the pattern of expression is different with a repression of *HSP α* and an induction of *TIP1* in leaves (Table 4). In both green stem and roots, *TIP1* was also up-regulated. In the green stems only, *GST1* and *CHV5* were up-regulated and *HSP α* strongly down-regulated (Table 4). Compared to 2015, most of the induction of genes reported have been observed again in 2016 (Table 4S). We could note that for *HSP α* , an inverse response in root at flowering and in leaves, green stems and root system at berry maturity was obtained. For the plants treated by ¼ rate of sodium arsenite, the pattern of the studied targeted genes is intermediate between the controls and plants full treated by sodium arsenite at both phenological stages (Table 4S).

4. Discussion

Our results confirmed that arsenic is translocated to all grapevine organs and accumulated in the soil. It is important to acknowledge that the treatment was applied in winter, at the end of rest period, when the plant was dormant. The spray was applied until runoff and invariably penetrated the soil. In our experiment, arsenic in the grafted Tempranillo has penetrated the bark and accumulated in all organs from roots to leaves. The detection in leaves demonstrates that arsenic moves through the plant via the vascular vessels. Many studies are concerned with the translocation of arsenic from roots to both shoots and leaves and it appears that the main form of arsenic found in xylem sap is inorganic arsenite As(III) (see Zhao et al., 2009 for review). Except for leaves, total arsenic concentration *in planta* was similar at both flowering and berry maturity stage probably because the grafted plants were in pots, a closed

system with limited drainage. For comparison, Carbonell-Barrachina et al. (1997a) reported that low total arsenic quantity was detected in leaves and also in fruits and their quantity decreased during the vegetative cycle in both Aledo and Italia cultivars. In another crop such as rice, total arsenic concentrations decrease also markedly in the order of roots > stems and leaves > fruit (Abedin et al., 2002; Liu et al., 2006; Xu et al., 2008). All the authors suggest that remobilization of arsenic from stems and leaves to other organs, if any, may be limited. Indeed, As(III) can form complexes with thiols like phytochelatins, which are sequestered in vacuoles, decreasing the As translocation (Raab et al., 2005). Moreover, efflux of arsenic to the soil from the root and vice versa could be involved. The high level of arsenic in the soil could result from the sodium arsenite treatment but also from the root exudation (Xu et al., 2007; Zhao et al., 2009 for review). Arsenic uptake pathway is well documented solely in rice by Ma et al. (2008). They reported that both Lsi1 and Lsi2 transporters were involved in influx and efflux of arsenic respectively. By the xylem vessels, a low quantity of arsenic is translocated in above-ground tissues and could be accumulated (Zhao et al., 2009; Quaghebeur and Rengel, 2004; Kofroňova et al., 2018).

The accumulation of arsenic alters the growth of the grafted plants especially the aerial organs. Compared to the controls, the **grapevines** treated with sodium arsenite showed a reduced growth in terms of the number, the length and the diameter of the green shoots especially at berry maturity. Amazingly, the development of the root system was affected by the treatment but not significantly. The root system inhibition could be correlated to the high concentration of arsenic detected in both the soil and the root system. The weak alteration of the root system could nevertheless have an impact on the development of the above-ground organs. **Such reduction of the plant growth was reported in field crops (Jacobs et al., 1970), especially in tomato (Carbonell-Barrachina et al., 1994; Carbonell-Barrachina et al., 1995), in bean (Carbonel-Barrachina et al., 1997b) and in rice (Marin et al., 1992, Zhao et al., 2009, Rahman et al., 2007).**

The reduction of the plant growth could be correlated to an alteration of the primary metabolism, especially the photosynthesis activity, due to the arsenic toxicity as reported by Kofroňova et al. (2018). In the present study and especially in 2015 experiment, A_n decreased significantly in sodium arsenite treated grafted vines during the 4 months after treatment (T+119 days). This decrease is correlated to a closure of stomata since g_s is low. Consequently, E is also limited. In opposite, C_i remains either stable or increased suggesting a

weak assimilation of the CO₂ by the RuBisCo enzyme. Srivastava et al. (2013) reported also that arsenic stress affects negatively the photosynthesis activity in *Hydrilla verticillate*, an aquatic weed, 96h after the treatment. The decrease of photosynthesis could be correlated to a decrease of photosynthetic pigments, chlorophyll-a and -b, as observed in *Oryza sativa* L. (Rahman et al., 2007). Six months after the sodium arsenite treatment, we observed inversely a significant increase of A_n, g_s and E with a later increase of Ci. ϕ PSII was firstly reduced in treated vines until T+119 days suggesting a stress and then it was similar between treated and control plants as described for the gas exchanges parameters. In the meantime, the quantity of arsenic increased in leaves from flowering to berry ripening suggesting that the accumulation of arsenic may stimulate the photosynthesis over time. This induction of the photosynthesis appeared too late to stimulate the plant growth since at berry maturity the plants treated by sodium arsenite were always less developed compared to controls. The decrease of photoassimilates due to a low photosynthesis the first four months and the probable low level of sugar storage inside the young grafted plants used (3-year-old) could explained the limitation of the growth. Differences noted at 3 months between sodium arsenite treated and control plants for growth were maintained at 6 months. In grapevine, an alteration of the photosynthetic mechanism was already reported after treatments with chemicals such as fludioxonil and fenhexamid (Petit et al., 2009, 2012) and by copper or sulfur (Palmer et al., 2003; Xia et al., 2006). The photosynthesis thus appears to be a crucial hub point to regulate toxicity responses under chemical stress including As. In our study, the impact of As could also result in the activation of some processes of detoxification and stress tolerance that may induce its accumulation in vacuole (Zhao et al., 2009, Gusman et al., 2013). To illustrate, the lettuce plants are able to accumulate sodium arsenite in leaves but also to protect the photosynthetic apparatus against As, probably through the activation of tolerance mechanisms (Gusman et al., 2013).

In complement to the alteration of both growth and photosynthetic activity, some processes of detoxification could be induced in sodium arsenite treated plants compared to controls, as suggested by Farooq et al. (2015, 2016) and reviewed by Kofroňova et al. (2018). In both leaves and green stems, the antioxidant system was altered as suggested by the **up-regulation** firstly of *SOD* at flowering, and then of *GST1* at berry maturity. In the meantime, *CHV5* encoding for a chitinase was up-regulated but only in green stem. For the root system, *GST1*, *CHV5* and *STS* were highly induced. All these strong inductions could be correlated to the higher level of arsenic content in roots and imply a reinforcement of stress tolerance

mechanisms and detoxification in presence of arsenic. Moreover, an oxidative stress was perceived by the vine since *GSTI* were mainly up-regulated and *SOD* up- or down-regulated according to the organs and the timepoint. For the antioxidant system, the enhanced activities of isoenzymes of superoxide dismutase, ascorbate peroxidase and glutathione reductase indicated that arsenic stress generates oxidative stress (Shri et al., 2009; Srivastava et al., 2013; Kumar et al., 2015 for review). Such plant responses to sodium arsenite were coupled with an antifungal growth activity correlated to both *STS* (Mazzullo et al., 2000; Coutos-Thévenot et al., 2001) and *CHV5* (Mauch et al., 1988) inductions. In the roots, the aquaporine TIP1 was also activated but only at berry maturity. Aquaporins belonging to NIPs (noduling-like intrinsic proteins) have the ability to transport multiple uncharged solutes including boric acid, salicylic acid (Wallace et al., 2006) as well as arsenic (Biernert et al., 2008, Ma et al., 2008). To date, it is supposed that some TIP channels may be permeable to arsenic and contribute to its transport in vacuole (Zhao et al., 2009). For the expression of *HSP α* , encoding for a heat shock protein, it was inversely up- and down-regulated in the plants at flowering and berry maturity, respectively. At now, it is known that arsenic may induce specific proteins expression in response to abiotic stresses such as a heat shock. Saltveit et al. (2004) observed a similar proteomic pattern between a heat shock and an arsenic stress in cucumber radicles. All these perturbations under arsenic stress have also been reported for other heavy metals or fungicides (for review Petit et al., 2012).

Comparing 2015 and 2016 experiments, the impact of a sodium arsenite treatment on plant physiology was globally the same. The alterations of the plant growth, the photosynthesis and the plant defense response in terms of detoxification process and stress tolerance were significantly higher in 2015 compared to 2016. Since the experiments were conducted under natural conditions, the weak differences between 2015 and 2016 could be explained by climatic conditions, slightly different between both years. The temperatures were thus globally lower in 2016 compared to 2015 while rainfall was higher (Figures 1S, 2S). Independently to the soil flushing that could result to the rainfalls, the evolution of the arsenic content in the soil from flowering to berry maturity are close in 2015 and 2016.

5. Conclusion

Until 2003 in Europe, sodium arsenite treatments were applied to control fungal diseases especially against pathogens associated to trunk diebacks in grapevine. Since the ban of the use of As, no similar efficient treatment is available to manage GTDs. The present study concludes that arsenic was translocated to leaves, the photosynthesis was firstly reduced and

then stimulated, and plant tolerance responses were induced including the antioxidant system. More in depth experiments focusing on arsenic stress on the plant microbiome may be required to know its global impact in the tripartite vine-GTDs pathogens-plant microbiome. The last step will be to identify a similar product for both plant and human health with the same efficacy as sodium arsenite to control GTDs by acting as a fungicide (Larignon et al., 2008) and plant elicitor as described herein.

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Figure captions

Figure 1. Arsenic content in leaves, trunk below the graft/bud union, roots and soil on control (T, black bars) and treated (A, gray bars) grafted-plants cv Tempranillo in 2016. Bar plain corresponds to the samples collected at flowering and bar with white points to the samples collected at berry maturity. Values $\pm$ SD are represented on the graph. Significant differences between control and treated plants for the different organs and for each collecting time are marked by * at $p < 0.05$, ** at $p < 0.01$ and *** at $p < 0.001$.

Figure 2. Changes in A, net photosynthesis (A_n); B, stomatal conductance (g_s); C, internal leaf CO_2 concentration (C_i); D, transpiration (E); E, photosystem II activity ($\Phi PSII$) and F, water use efficiency (WUE) as A_n/g_s , measured in leaves of control (black squares) and treated (grey squares) plant cv Tempranillo by sodium arsenite in 2015. Measurements were performed at different dates from before the flowering, 67 days after the treatment with sodium arsenite, to the berry maturity, 167 days after the treatment. The mean $\pm$ SE was calculated from 5 biological replicates. Significant differences between control and treated plants are marked by * at $p < 0.05$, ** at $p < 0.01$ and *** at $p < 0.001$.

Table 1. Primers of genes analyzed by real-time reverse-transcription polymerase chain reaction. *TC TIGR: tentative consensus sequences

Function	Gene	Primer Sequences	GenBank or TC TIGR Accession Number
Housekeeping genes	<i>UBE2</i> (ubiquitin carrier protein E2)	5'-CATCCTTCAGGACAAGTGGT-3' 5'-GAGCTTTACAGATATACGAAACCG-3'	XM_002275879.1
	<i>EF1-α</i> (elongation factor 1- α)	5'-GAACTGGGTGCTTGATAGGC-3' 5'-AACCAAAATATCCGGAGTAAAGA-3'	GU585871
	<i>60SRP</i> (60S ribosomal protein L18)	5'-ATCTACCTCAAGCTCCTAGTC-3' 5'-CAATCTTGTCCTCCTTCCT-3'	XM_002270599
	<i>STS</i> (stilbene synthase)	5'-AGGAAGCAGCATTGAAGGCTC-3' 5'-TGCACCAGGCATTTCTACACC-3'	FJ851185
Defense protein	<i>CHV5</i> (class V chitinase)	5'-CTACAACTATGGCGCTGCTG-3' 5'-CCAAAACCATAATGCGGTCT-3'	AF532966
	<i>PR1</i> (pathogenesis-related protein 1)	5'-GGAGTCCATTAGCACTCCTTTG-3' 5'-CATAATTCTGGGCGTAGGCAG-3'	XM_002273752.2
Detoxification and stress tolerance	<i>GST1</i> (glutathion-S-transferase 1)	5'-TGCAATGGAGGAGGAGTTCGT-3' 5'-CAAGGCTATATCCCCATTTCTTC-3'	AY156048
	<i>HSPα</i> (alpha crystalline heat shock protein)	5'-TCGGTGGAGGATGACTTGCT-3' 5'-CGTGTGCTGTACGAGCTGAAG-3'	XM_002272382
	<i>SOD</i> (superoxide dismutase)	5'-GTGGACCTAATGCAGTGATTGGA-3' 5'-TGCCAGTGGTAAGGCTAAGTTCA-3'	AF056622
	<i>TIP1</i> (tonoplast intrinsic aquaporin)	5'-ATCACCACCTCATTTCATATGC-3' 5'-GTTGTTGTCTCAACCCATTTC-3'	AF271661

Table 2. Arsenic contents (mean value $\pm$ SD) in mg/kg DW measured in 2015 and 2016 in leaves, trunk below the graft/bud union, roots and soil of the grafted grapevine cv. Tempranillo untreated (C) and treated (full, As and one quarter of the prior application rate, As $\frac{1}{4}$). Significant differences between the three modalities (As, As $\frac{1}{4}$ and C) for each area are indicated by letters (if letters are different, the difference between groups are significant). Nd: not determined.

		Leaves			Trunk			Roots			Soil		
		C	As 1/4	As	C	As 1/4	As	C	As 1/4	As	C	As 1/4	As
2016	Flowering (mean)	0.01 a	0.91 b	2.35 c	0.48 a	1.72 b	3.94 b	0.54 a	5.15 b	10.44 c	1.22 a	7.17 b	15.62 c
	Flowering SD	0.00	0.40	0.68	0.20	0.64	1.81	0.18	3.35	4.27	0.23	3.48	5.97
	Berry maturity (mean)	0.19 a	5.26 b	9.00 c	0.43 a	3.25 b	7.46 c	0.60 a	5.02 b	14.35 c	1.48 a	19.64 b	33.75 b
	Berry maturity SD	0.12	1.20	1.76	0.17	1.91	3.31	0.23	1.45	9.29	0.64	12.85	31.01
2015	Flowering (mean)	0.12 a	nd	3.42 b	nd	nd	nd	0.48 a	nd	9.80 b	nd	nd	nd
	Flowering SD	0.00	nd	0.64	nd	nd	nd	0.02	nd	1.27	nd	nd	nd

Table 3. Growth parameters of the grafted grapevine cv. Tempranillo untreated (Control) and treated by sodium arsenite (Treated) measured at flowering and at berry maturity in 2015. Values are means $\pm$ SE of 5 biological replicates. Significant differences between each treated condition and the controls are marked by * at $p < 0.05$, ** at $p < 0.01$, *** at $p < 0.001$.

	Flowering		Berry maturity	
	Control	Treated	Control	Treated
Number of green shoots	3.00 $\pm$ 0.45	2.00 $\pm$ 0.32	4.20 $\pm$ 0.37	2.40 $\pm$ 0.40*
Length of green shoots (cm)	35.60 $\pm$ 1.03	12.60 $\pm$ 3.86**	50.30 $\pm$ 4.31	26.00 $\pm$ 5.82*
Diameter of green shoots (cm)	0.81 $\pm$ 0.04	0.60 $\pm$ 0.04**	0.80 $\pm$ 0.05	0.51 $\pm$ 0.03**
Fresh weight of root system (g)	209.08 $\pm$ 43.54	168.67 $\pm$ 36.59	318.73 $\pm$ 50.01	286.86 $\pm$ 30.38
Volume of root system (cm ³)	216.78 $\pm$ 42.10	163.58 $\pm$ 30.28	366.83 $\pm$ 45.86	278.70 $\pm$ 34.03

Table 4. Relative expression levels of the 6 selected genes recorded by RT-qPCR in leaves, green stems and roots of grafted grapevines cv. Tempranillo treated with sodium arsenite at flowering and at berry maturity in 2015, i.e. 90 and 183 after the treatment respectively. Values correspond to the means $\pm$ SE of 5 biological replicates. Gene expression of untreated plant (control) was used as reference to calculate relative expression. A given gene was considered up- or down-regulated when the value of relative expression was >2 -fold or <0.5 -fold compared to the controls, respectively. Genes down- or over-expressed appear in shades of grey, with expression levels lower or higher than 0.1 and 30, respectively, in dark grey. In white, there is no change in gene expression compared to the controls. nd: not determined; ne: not expressed.

	Flowering			Berry maturity		
	Leaves	Green stems	Roots	Leaves	Green stems	Roots
<i>GST1</i>	ne	ne	64.95 $\pm$ 19.81	21.03 $\pm$ 2.86	6.25 $\pm$ 2.72	6.90 $\pm$ 2.90
<i>SOD</i>	26.86 $\pm$ 2.04	2.7 $\pm$ 0.19	1.03 $\pm$ 0.06	0.63 $\pm$ 0.13	0.97 $\pm$ 0.05	1.09 $\pm$ 0.21
<i>HSPα</i>	2.63 $\pm$ 0.40	0.73 $\pm$ 0.10	3.28 $\pm$ 0.73	0.29 $\pm$ 0.00	0.08 $\pm$ 0.02	0.42 $\pm$ 0.12
<i>CHV5</i>	0.56 $\pm$ 0.16	4.18 $\pm$ 2.23	9.79 $\pm$ 3.95	0.60 $\pm$ 0.14	16.96 $\pm$ 7.23	6.32 $\pm$ 2.25
<i>STS</i>	nd	4.59 $\pm$ 1.27	5.47 $\pm$ 1.77	nd	1.3 $\pm$ 0.41	2.04 $\pm$ 0.69
<i>TIP1</i>	0.52 $\pm$ 0.10	0.28 $\pm$ 0.01	0.83 $\pm$ 0.11	2.43 $\pm$ 0.22	2.01 $\pm$ 0.14	7.26 $\pm$ 0.67

≤ 0.1 0.5 2 ≥ 30

Supplementary material

Table 1S. ICP-MS operating parameters

RF Power	1550 W
Carrier gas	0.9 Lmin ⁻¹
Make-up gas	0.21 L min ⁻¹
Collision gas	He (5 mL min ⁻¹)
Spray chamber temperature	2°C
Detector mode	Pulse Counting
Integration time	0.3 s

Table 2S. Growth parameters of the grafted grapevine cv. Tempranillo untreated (Control) and treated by sodium arsenite (full, As and one quarter of the prior application rate, As ¼) measured at flowering and at berry maturity in 2016. Values are means $\pm$ SE of 10 biological replicates. Significant differences between each treated condition and the controls are marked by * at $p < 0.05$, ** at $p < 0.01$, *** at $p < 0.001$.

	Flowering			Berry maturity		
	Control	As 1/4	As	Control	As 1/4	As
Number of green shoots	4.40 $\pm$ 0.48	2.90 $\pm$ 0.41*	2.90 $\pm$ 0.23*	3.20 $\pm$ 0.29	2.40 $\pm$ 0.22*	2.70 $\pm$ 0.21
Length of green shoots (cm)	37.31 $\pm$ 3.12	39.86 $\pm$ 3.25	30.27 $\pm$ 4.57	50.03 $\pm$ 5.56	47.13 $\pm$ 4.48	32.80 $\pm$ 5.25*
Diameter of green shoots (cm)	0.47 $\pm$ 0.02	0.47 $\pm$ 0.02	0.43 $\pm$ 0.03	0.55 $\pm$ 0.03	0.54 $\pm$ 0.02	0.47 $\pm$ 0.03
Fresh weight of root system (g)	203.53 $\pm$ 24.14	207.30 $\pm$ 24.07	199.36 $\pm$ 23.58	192.69 $\pm$ 22.57	179.87 $\pm$ 36.71	224.72 $\pm$ 29.08
Volume of root system (cm ³)	181.63 $\pm$ 13.47	185.93 $\pm$ 19.82	192.95 $\pm$ 19.86	187.89 $\pm$ 27.92	168.70 $\pm$ 32.31	214.37 $\pm$ 27.44

Table 3S. Changes in net photosynthesis (A_n), stomatal conductance (g_s), internal leaf CO_2 concentration (C_i), transpiration (E) and water use efficiency (WUE) as A_n/g_s measured in leaves of grafted grapevine cv. Tempranillo untreated (Control) or treated by sodium arsenite in 2016 (full, As and one quarter of the prior application rate, As $1/4$). The mean $\pm$ SE was calculated from 10 biological replicates at different dates from before the flowering, 96 days after the treatment with sodium arsenite, to the berry maturity, 157 days after the treatment date. Significant differences between each treated conditions and the controls are marked by * at $p < 0.05$, ** at $p < 0.01$, *** at $p < 0.001$.

Days post treatment	Control	As 1/4	As
A_n ($\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$)			
96	6.34 $\pm$ 0.30	6.49 $\pm$ 0.24	5.69 $\pm$ 0.15 *
109	5.38 $\pm$ 0.23	5.66 $\pm$ 0.27	5.59 $\pm$ 0.38
130	2.86 $\pm$ 0.36	4.19 $\pm$ 0.55 *	2.99 $\pm$ 0.25
136	4.59 $\pm$ 0.49	4.54 $\pm$ 0.34	4.28 $\pm$ 0.43
157	3.45 $\pm$ 0.43	3.77 $\pm$ 0.43	3.09 $\pm$ 0.54
g_s ($\text{mol H}_2\text{O m}^{-2}\text{s}^{-1}$)			
96	0.13 $\pm$ 0.01	0.14 $\pm$ 0.01	0.09 $\pm$ 0.01 ***
109	0.11 $\pm$ 0.02	0.14 $\pm$ 0.01	0.10 $\pm$ 0.01
130	0.05 $\pm$ 0.00	0.06 $\pm$ 0.01	0.03 $\pm$ 0.00 **
136	0.08 $\pm$ 0.01	0.10 $\pm$ 0.01	0.07 $\pm$ 0.01
157	0.08 $\pm$ 0.01	0.09 $\pm$ 0.01	0.07 $\pm$ 0.01
C_i ($\mu\text{mol CO}_2 \text{ mol}^{-1}$)			
96	292.22 $\pm$ 2.11	291.58 $\pm$ 4.01	246.70 $\pm$ 17.03 *
109	261.18 $\pm$ 29.56	302.28 $\pm$ 3.96	275.06 $\pm$ 7.94
130	280.27 $\pm$ 11.37	246.54 $\pm$ 11.71 *	203.83 $\pm$ 17.31 **
136	279.36 $\pm$ 4.47	290.68 $\pm$ 6.11	264.85 $\pm$ 9.91
157	303.10 $\pm$ 9.93	294.94 $\pm$ 5.83	297.15 $\pm$ 7.28
E ($\text{mmol H}_2\text{O m}^{-2}\text{s}^{-1}$)			
96	1.64 $\pm$ 0.08	1.55 $\pm$ 0.09	1.18 $\pm$ 0.11 **
109	1.40 $\pm$ 0.14	1.65 $\pm$ 0.11	1.30 $\pm$ 0.08
130	0.97 $\pm$ 0.08	1.22 $\pm$ 0.13	0.73 $\pm$ 0.06 *
136	1.21 $\pm$ 0.11	1.50 $\pm$ 0.10 *	1.16 $\pm$ 0.12
157	1.32 $\pm$ 0.16	1.65 $\pm$ 0.14	1.23 $\pm$ 0.22
WUE ($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)			
96	48.96 $\pm$ 1.28	48.83 $\pm$ 2.12	77.46 $\pm$ 10.83 **
109	52.29 $\pm$ 3.65	43.83 $\pm$ 2.49 *	60.56 $\pm$ 4.96
130	55.71 $\pm$ 7.16	76.45 $\pm$ 7.39 *	103.22 $\pm$ 10.97 **
136	57.37 $\pm$ 2.80	50.08 $\pm$ 3.87	66.12 $\pm$ 6.26
157	41.97 $\pm$ 6.17	46.04 $\pm$ 3.66	44.83 $\pm$ 4.81

Table 4S. Relative expression levels of the 6 selected genes recorded by RT-qPCR in leaves, green stems and roots of grafted grapevines cv. Tempranillo treated with sodium arsenite (full, As and one quarter of the prior application rate, As $\frac{1}{4}$) at flowering and at berry maturity in 2016, i.e. 64 and 188 after the treatment respectively. Values correspond to the means $\pm$ SE of 10 biological replicates. Gene expression of untreated plant (control) was used as reference to calculate relative gene expression for the treated plant (As and As $\frac{1}{4}$). A given gene was considered up- or down-regulated when the value of relative expression was >2 -fold or <0.5 -fold compared to the control, respectively. Genes down- or over-expressed appear in shades of grey, with expression levels lower or higher than 0.1 and 30, respectively, in dark grey. In white, there is no change in gene expression compared to the control. ND: not determined.

	Flowering						Berry maturity					
	Leaves		Green stems		Roots		Leaves		Green stems		Roots	
	As	As $\frac{1}{4}$	As	As $\frac{1}{4}$	As	As $\frac{1}{4}$	As	As $\frac{1}{4}$	As	As $\frac{1}{4}$	As	As $\frac{1}{4}$
<i>GST1</i>	1.53 $\pm$ 0.12	1.09 $\pm$ 0.37	3.38 $\pm$ 0.59	1.00 $\pm$ 0.17	8.60 $\pm$ 3.40	6.18 $\pm$ 2.04	6.22 $\pm$ 1.67	2.56 $\pm$ 0.45	0.35 $\pm$ 0.08	1.87 $\pm$ 0.55	0.81 $\pm$ 0.44	1.69 $\pm$ 0.33
<i>SOD</i>	0.90 $\pm$ 0.12	2.36 $\pm$ 0.21	1.39 $\pm$ 0.07	1.22 $\pm$ 0.08	0.77 $\pm$ 0.10	1.06 $\pm$ 0.22	1.11 $\pm$ 0.15	0.67 $\pm$ 0.08	1.05 $\pm$ 0.10	0.83 $\pm$ 0.04	0.57 $\pm$ 0.18	0.50 $\pm$ 0.02
<i>HSPα</i>	9.51 $\pm$ 3.19	2.91 $\pm$ 0.58	1.41 $\pm$ 0.10	1.73 $\pm$ 0.26	0.26 $\pm$ 0.10	0.45 $\pm$ 0.06	72.23 $\pm$ 8.46	1.16 $\pm$ 0.21	11.22 $\pm$ 4.39	1.45 $\pm$ 0.35	17.75 $\pm$ 5.15	40.73 $\pm$ 0.41
<i>CHV5</i>	0.73 $\pm$ 0.06	0.85 $\pm$ 0.05	1.11 $\pm$ 0.26	0.64 $\pm$ 0.09	3.53 $\pm$ 0.51	1.80 $\pm$ 0.41	2.17 $\pm$ 0.53	3.37 $\pm$ 0.47	0.75 $\pm$ 0.14	0.86 $\pm$ 0.18	2.94 $\pm$ 0.46	1.95 $\pm$ 0.45
<i>STS</i>	0.92 $\pm$ 0.07	0.58 $\pm$ 0.13	3.77 $\pm$ 0.72	0.55 $\pm$ 0.08	2.12 $\pm$ 0.62	1.26 $\pm$ 0.23	ND	ND	0.59 $\pm$ 0.13	0.78 $\pm$ 0.17	0.75 $\pm$ 0.13	2.62 $\pm$ 0.59
<i>PR1</i>	0.46 $\pm$ 0.10	1.30 $\pm$ 0.16	1.19 $\pm$ 0.32	2.43 $\pm$ 0.32	2.31 $\pm$ 1.17	2.95 $\pm$ 0.73	1.50 $\pm$ 0.19	2.68 $\pm$ 0.27	0.39 $\pm$ 0.12	0.48 $\pm$ 0.12	0.07 $\pm$ 0.02	0.28 $\pm$ 0.07

Figure 1.

Figure 2.

GTD foliar
Symptoms inhibition

Sodium arsenite
treatment in winter

2 sampling periods:
Flowering
Berry maturity

Reduction of
growth

Alteration of
photosynthesis and
plant defense
responses

Quantification As