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The *in Vitro* Effect of the Herbicide Basta® (Glufosinate Ammonium) on Potential Fungal Grapevine Pathogens

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Summary

Glufosinate ammonium-based herbicides are commonly used in commercial vineyards to control excessive growth of weeds. However, glufosinate ammonium also seems to have antifungal properties. Since recent reports indicate fungicidal activities also to grape pathogens, several fungal organisms putatively pathogenic to grapevine (*Botrytis cinerea*, *Guignardia bidwellii*, *Penicillium expansum*, *Phomopsis viticola*) were exposed to glufosinate ammonium in an *in vitro* assay. The herbicidal compound caused reduction of mycelial growth in a dose dependent manner. *G. bidwellii* was the most sensitive pathogen, and mycelial growth was reduced about of 80 % when exposed to a 500fold diluted solution of glufosinate ammonium normally applied to the field. As an interesting side effect, glufosinate ammonium nearly inhibited spore production in *Penicillium expansum* cultures when exposed to the same low concentration. Even though fermentation of must by yeasts may be affected due to residues attached to grapes, spray applications of glufosinate ammonium to the ground leading to a dispersal of small amounts to non-treated areas could help to reduce the built-up of secondary inoculum in the same season or primary inoculum for the following year.

Key words. black rot – fungi – green mould – grey mould – *Vitis vinifera*

Introduction

Agricultural chemicals are widely used to improve growth and development of crop plants. Even though they are used for specific purposes, such as inhibiting fungal infections (fungicides), preventing damage by insects (insecticides) or excessive growth of weeds (herbicides), the active ingredients frequently induce side effects on their target crops and agroecosystems. For example, some triazole fungicides act as growth regulators (Buchenauer 1995) and can also protect plants against various stresses, including drought or flooding stress (Lin et al. 2006), low and high temperatures, and UV radiation (Fletcher et al. 2000). Furthermore, applications of strobilurin-based fungicides resulted in greater yields and quality benefits due to the 'greening effect', which means that treated crops maintain green leaves until late in the season, thereby maximising biomass production (Bartlett et al. 2002). On the other side, some herbicides are known to induce morphological or physiological alterations in crops leading to an enhanced or decreased susceptibility to plant diseases. Glyphosate occasionally suppresses disease defence responses and increases disease development mainly due to the inhibition of secondary plant products such as phytoalexins. For example, reduced phytoalexin levels increased susceptibility to *Alternaria* (Sharon et al. 1992), *Verticillium* (Latunde-Dada and Lucas 1985), *Colletotrichum* (Johal and Rahe 1988) or to oomycetes (Ward 1984; Liu et al. 1997), and glyphosate was thus tested as a synergist for microbial weed biocontrol products (Christy et al. 1993). Neumann et al. (2006) proposed that glyphosate could also reduce resistance to pathogens by limiting micronutrient availability. This agrees with the observation that glyphosate increases the susceptibility to root diseases (Larson et al. 2006). However, herbicides with other mechanisms of action can stimulate the production of fungitoxic compounds and thereby influence plant disease resistance (Grinstein et al. 1984; Tamogami et al. 1995). High levels of the phytoalexin glyceollin are induced by lactofen in soybeans, resulting in some protection from white mould caused by *Sclerotinia* (Dann et al. 1999). Furthermore, herbicides can be directly toxic to plant pathogens at rates that are applied to crops, even though the fungicidal activity may have a different mechanism than the herbicidal activity in some cases. Effects on fungal pathogens can be either through killing the spore or preventing its germination, or inhibiting mycelial growth and sporulation when herbicides are used at field rates (Wyss et al. 2004; Yandoc et al. 2006). The development of herbicide-resistant crops has enabled the evaluation of disease control effects without herbicidal crop injury. Recent studies showed that glyphosate is able to reduce incidences of rusts in glyphosate-resistant soybean and wheat (Anderson and Kolmer 2005; Feng et al. 2008). Interestingly, glyphosate has also been reported to control mammalian pathogenic fungi (Nosanchuk et al. 2001) and was active against apicomplexan parasites that cause diseases such as malaria

and toxoplasmosis (Roberts et al. 2002). Besides glyphosate, the herbicide glufosinate or glufosinate ammonium also exhibits toxic activities against several fungal pathogens and is able to induce defense responses. Its antifungal effects on pathogens are best seen in glufosinate-resistant crops, such as rice (Ahn 2008) and creeping bentgrass (Wang et al. 2003), since higher doses could be applied. The non-selective herbicide glufosinate ammonium is an ammonium salt of phosphinothricin and efficiently kills various kinds of plants, since it is a glutamic acid analog that inhibits glutamine synthetase by irreversible binding (Hoerlein 1994). The inhibition of glutamine synthetase results in an accumulation of toxic ammonium that disturbs electron transport systems and induces production of free radicals (Krogmann et al. 1959). Free radicals in turn cause lipid peroxidation and cell death (Hess 2000). Interestingly, applications of sublethal doses of glufosinate ammonium could reduce downy mildew infestations on grapevine without negatively affecting the host plant (Kortekamp 2008). Since glufosinate ammonium is commonly used to control weeds in the vineyard, spray applications leading to a drift of the herbicide to non-treated areas surely interfere with the grape's state of health by causing direct toxic effects (Schildberger et al. 2007) or by lowering infections caused by pathogens. In spite of the fact that glufosinate ammonium targets a primary pathway shared commonly by plants and microorganisms, nothing is known about its effect on potential grape pathogens, such as grey mould caused by *Botrytis cinerea* or green mould caused by *Penicillium expansum*, especially when applied at sub-lethal levels. Therefore, the effect of glufosinate ammonium on fungal pathogens putatively infecting grapes was investigated *in vitro* in order to demonstrate a more general inhibitory capacity of this herbicidal agent. For this purpose, several concentrations of glufosinate ammonium had been tested, especially those concentrations considered to be non-toxic to organisms.

Material and Methods

Cultures of *Botrytis cinerea*, *Guignardia bidwellii*, *Penicillium expansum*, and *Phomopsis viticola* represent field isolates. They were kindly supported by M. Harms (DLR Rheinpflalz, Neustadt/Weinstraße, Germany) and maintained on malt extract agar (2 % w/w malt extract, 1.8 % w/w agar, both from AppliChem, Darmstadt, Germany) and on potato dextrose agar (3.9 % w/w Roth, Karlsruhe, Germany), respectively. For radial growth inhibition studies, glufosinate ammonium was incorporated as the formulated herbicide Basta® [200 g l^{-1} glufosinate ammonium, soluble concentration (SL); Bayer CropScience, Frankfurt, Germany] into the appropriate agar medium while still warm after autoclaving to give various concentrations. Mycelial plugs were cut from fresh cultures grown on control agar media using a cork borer and aseptically transferred to the centre of the agar plates containing glufosinate ammonium. Plates containing medium without pesticide served as controls. Test plates were sealed with parafilm and incubated at 25 °C under day light conditions. There were 10 plates for each treatment with 3 independent replications. Data were collected after 4 (*B. cinerea*) or 12 days (*G. bidwellii*, *P. expansum* and *Ph. viticola*) of incubation, as all test organisms on the control plates nearly covered the medium by that time. Growth was measured as the diameter of the colonies using a precise ruler. Preliminary experiments had shown that in case for *P. expansum* mainly the spore production was affected. Therefore, spore production was investigated according to Kortekamp 2006: Complete cultures were removed from the dishes, transferred into Erlenmeyer flasks containing 50 ml bidistilled water and 50 µl Tween 80®, and shaken on a rotary shaker at 250 rpm for 30 min. Aliquots of 1 ml were diluted to appropriate concentrations and spore densities were determined using a Fuchs- Rosenthal hemacytometer. Each experimental unit consisted of 5–10 Petri dishes or flasks for each concentration and the control. Three to four experimental units were performed.

To test if small amounts of glufosinate ammonium may interfere with must fermentation, 150 mg dry yeast (Oenoferm® Rouge, Erbslöh, Geisenheim, Germany) was suspended in 750 µl of sterile grape juice and incubated for 30 min at 37 °C. Then, 300 ml of sterile grape juice were inoculated with 225 µl of the freshly prepared yeast suspension. Aliquots of 25ml were transferred to 100ml Erlenmeyer flasks, amended with glufosinate ammonium to give various concentrations, and incubated at 20°C. Must density was determined daily under axenic conditions over a 10-day period using a Master-T refractometer (Kübler GmbH, Karlsruhe, Germany) and expressed as °Oe.

For the statistical analysis, all results were tested for significant differences. All analyses were carried out using SPSS 15.0 (SPSS GmbH Software München, Germany). The analysis of variance (ANOVA) with the multiple comparison test of Tukey ($P \leq 0.05$) was conducted in order to produce multiple, pairwise comparisons among means.

Results

Direct toxic effects of glufosinate ammonium towards pathogenic fungi were tested on Petri dishes amended with the herbicide Basta®. Preliminary experiments had shown that there was no difference between the formulated herbicide and the pure herbicidal compound regarding its effect on growth and spore production. Hence, all experiments were performed with the commercial formulated chemical. In the absence of glufosinate ammonium, the fungi tested grew equally well on the respective medium in all separate experiments. However, glufosinate ammonium caused severe reduction of mycelial growth, depending on concentration and species. Radial growth of all pathogens decreased with increasing concentrations of glufosinate ammonium. In general, *B. cinerea* showed the fastest mycelial growth and appeared to be less sensitive against the herbicide. However, radial growth was significantly reduced at the lowest concentration tested (0.05 mM). Even though there was no enhanced growth reduction caused by glufosinate ammonium at concentrations ranging from 0.1 to 0.3125 mM (Fig. 1a), radial growth decreased dramatically at concentrations used for field applications (5 to 25 mM), suggesting a toxic effect towards *B. cinerea* in the field when treated directly. The concentration at which radial growth was reduced to 50 % of the control's growth rate (EC_{50}) was about 0.625 mM. Radial growth of *Ph. viticola* was similarly affected (Fig. 1b), even

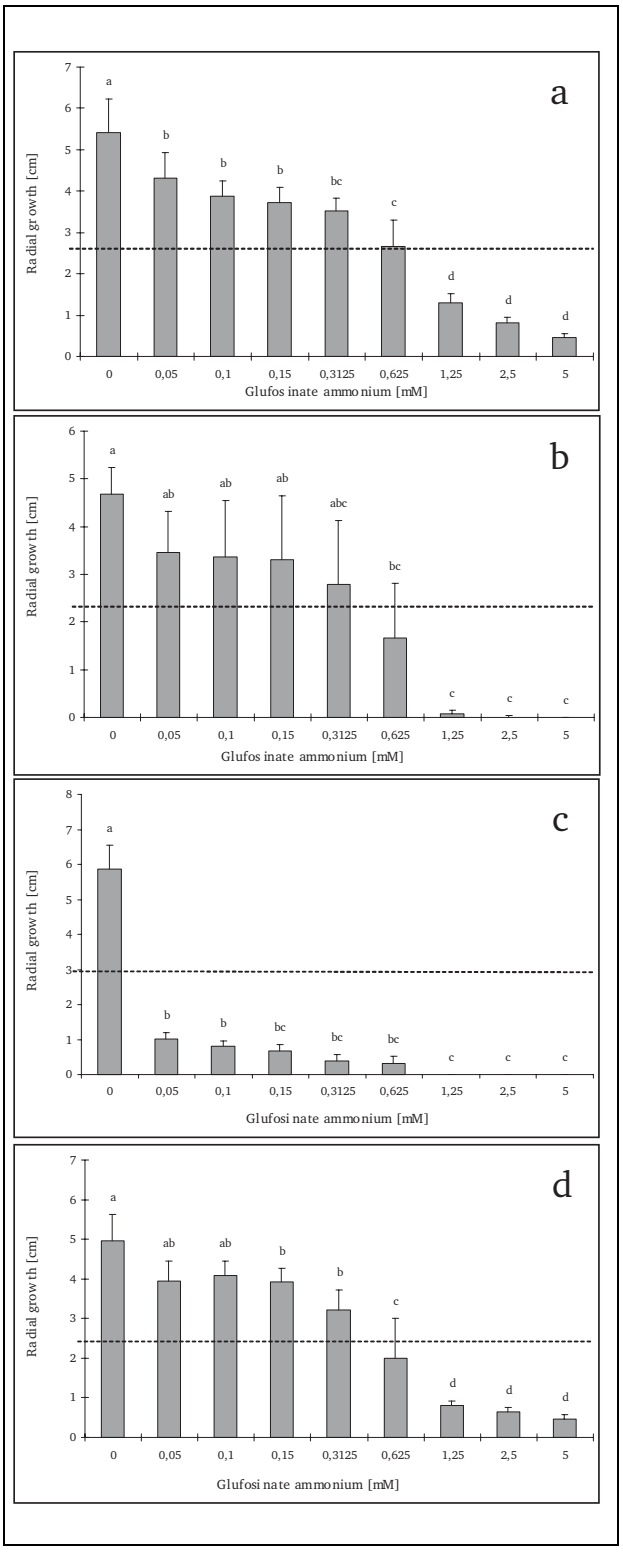


Fig. 1. Mycelial growth of *Botrytis cinerea* (a, 4 d post inoculation), *Phomopsis viticola* (b, 12 d post inoculation), *Guignardia bidwellii* (c, 12 d post inoculation), and *Penicillium expansum* (d, 12 d post inoculation) on artificial media amended with glufosinate ammonium (Basta®). Dotted lines represent the EC_{50}, the concentration at which radial growth is reduced to 50 % of the control's growth rate. Small letters indicate significant difference (Tukey-test, P=0.05).

though growth rate differed not significantly from that of the control when exposed to glufosinate ammonium at concentrations ranging from 0.05 to 0.3125 mM. However, the EC_{50} seemed to be below 0.625 mM and total growth inhibition was observed at 1.25 mM. In addition, a deformation of hyphae and browning of the mycelium occurred when the fungus was exposed to a concentration of at least 0.15 mM glufosinate ammonium (not shown). The pathogen *G. bidwellii* was the most sensitive fungus regarding its growth inhibition caused by glufosinate ammonium when compared to all the other fungi tested. Radial growth was reduced of about 80 % when exposed to the lowest concentration of 0.05 mM glufosinate ammonium and totally inhibited at concentrations exceeding 0.625 mM (Fig. 1c). Thus, the EC_{50} could not be exactly determined but seemed to be below 0.05 mM glufosinate ammonium. In the presence of glufosinate ammonium, the white aerial hyphae of *G. bidwellii* appeared only in small areas around the inoculation plugs. This was not the case for *P. expansum* that showed a normal growth when exposed to low glufosinate ammonium concentrations (0.05 and 0.1 mM glufosinate ammonium). Radial growth was only significantly reduced when treated with concentrations exceeding 0.15 mM (Fig. 1d) and the EC_{50} was below 0.625 mM glufosinate ammonium. However, as seen from Fig. 2a, spore production was nearly inhibited at any glufosinate ammonium concentration tested. To quantify the inhibition of spore production, cultures of *P. expansum* were transferred to a diluted Tween 80® solution in Erlenmeyer flasks, and the spores were removed from the mycelium by shaking on a rotary shaker and counted in a hemacytometer. Even though there was no significant reduction of mycelial growth at low concentrations of glufosinate ammonium as mentioned above, spore production of this fungus was nearly completely inhibited (Fig. 2b). An exposure of *P. expansum* to 0.05 mM glufosinate ammonium reduced spore production to less than 1 % compared to the non-treated control. Spore production of the other fungi tested was not or only slightly affected in case for low herbicide concentrations (not shown).

The results obtained with several fungi indicated that the inhibitory effect differed depending on the concentration and species used. Since low amounts of glufosinate ammonium may still adhere on collected berries if herbicides such as Basta® had been used in the vineyard at late time points and may thus contaminate the must, the effect of this herbicidal compound on fermentation by yeasts was also tested (Fig. 3). The fermentation of grape juice was not significantly affected using glufosinate concentrations up to 0.01 mM, but was significantly delayed at concentrations of more than 0.01 mM. A delay of fermentation increased with increasing concentrations of glufosinate ammonium. The onset of fermentation was delayed of about one day at a concentration of 0.05 mM glufosinate ammonium and of about four days at a concentration of 0.1 mM glufosinate ammonium. There was no fermentation detectable at concentrations exceeding 0.1 mM glufosinate ammonium.

Discussion

Herbicides are generally applied to control weeds in agricultural soils used for crop production. However, there is

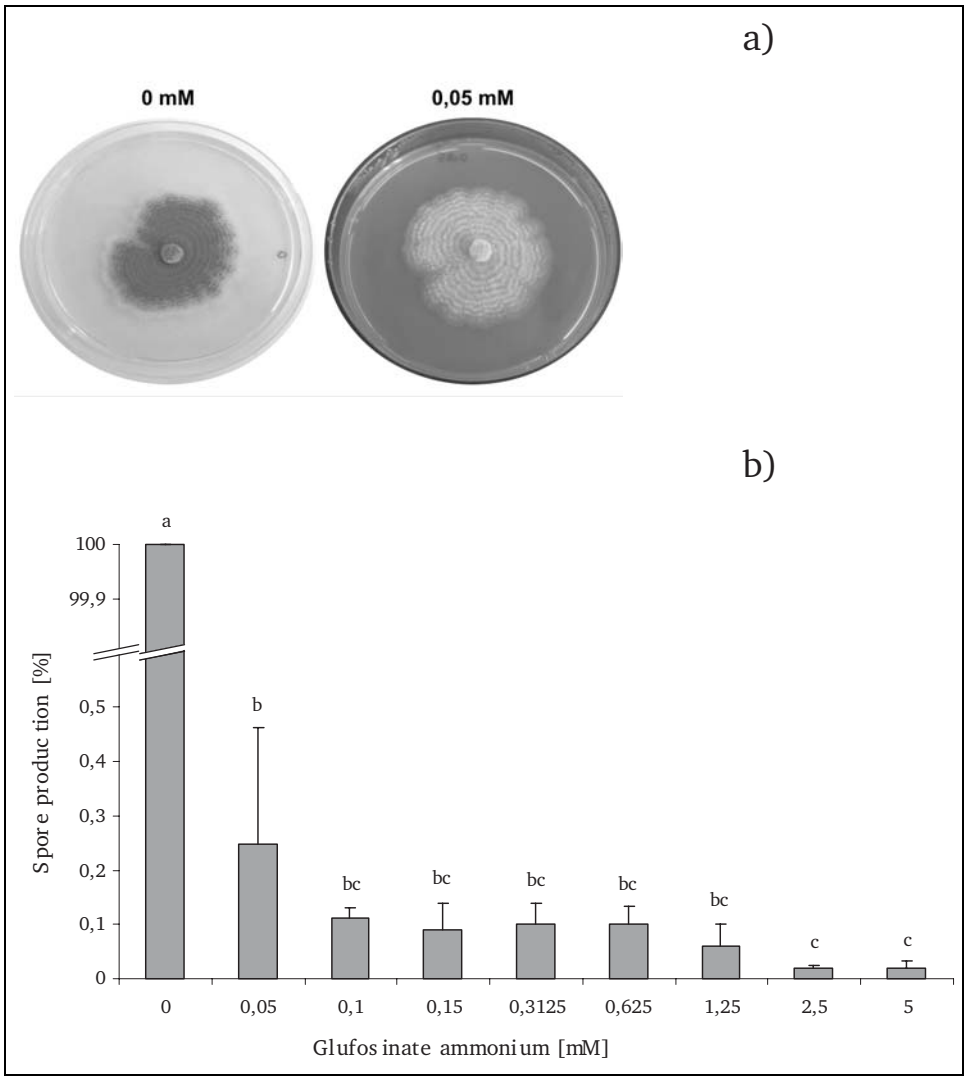


Fig. 2. Effect of glufosinate ammonium (Basta®) on spore production of *Penicillium expansum* on artificial medium. Incorporation of glufosinate ammonium to the medium lead to a visible decoloration of the culture due to an inhibited spore production (a), which was verified by an exact determination of spore numbers (b). Small letters indicate significant difference (Tukey-test, P=0.05).

the possibility that the activity of herbicides may extend beyond their target organisms. Some herbicides have the potential to alter plant susceptibility to diseases, such that they become more or less resistant to infections by pathogens. As another effect, herbicides may predispose pathogens to effects of fungicides and may thus increase the efficacy of the fungicide via direct synergism. Since amino acid biosynthesis is the primary target of glufosinate ammonium, inhibition of the enzyme glutamine synthetase leads to a reduced nitrogen metabolism. A reduced hyphal protein content and a restricted growth of fungi, as observed in *Trichoderma* species (Ahmad et al. 1995), may weaken fungal pathogens and improve the control by defence mechanisms of the host plant. Both factors, increased susceptibility to fungicides provoked by a direct toxic effect of herbicides and the plant's defence response, may enhance crop resistance against fungal pathogens. Unfortunately, very little is known about the interaction of herbicides with fungi infecting grapevine. Growth and development of *Plasmopara viticola*, the downy mildew pathogen of grapevine, was clearly reduced or inhibited *in planta* by sublethal doses of glufosinate ammonium (Kortekamp 2008) leading to a reduced infestation in the field (Albrecht and Kortekamp 2008). However, since *P. viticola* is an obligate biotrophic pathogen not able to survive and fulfil its life cycle without its host plant, it is not known if glufosinate ammonium affects *P. viticola* directly or stimulates the plant's defence response. It was recently shown that closely related species such as *Phytophtora nicotianae* expresses glutamine synthetase at an elevated level when germinating (Shan et al. 2004), suggesting a direct toxic effect by inhibiting amino acid biosynthesis. Even though a direct toxic activity against fungal pathogens is described for glufosinate ammonium (Wang et al. 2003) applied to transgenic plants at field doses, nothing is known about low-dose effects to grape pathogens mainly infecting berries. All fungi tested so far showed significantly reduced growth rates suggesting direct toxic activities of glufosinate ammonium to various fungi. Since antifungal effects could be observed in case for low doses of the herbicide, small amounts dispersed to non-treated areas by wind currents and residues on leaves and berries could reduce the probability of fungal infections. This could especially be the case for *G. bidwellii* and *P. expansum*. Both fungal pathogens seem to be highly sensitive regarding growth or spore production when getting in contact with low amounts of the herbicide. *Guignardia bidwellii* is the causal agent of black rot of grape, which is a

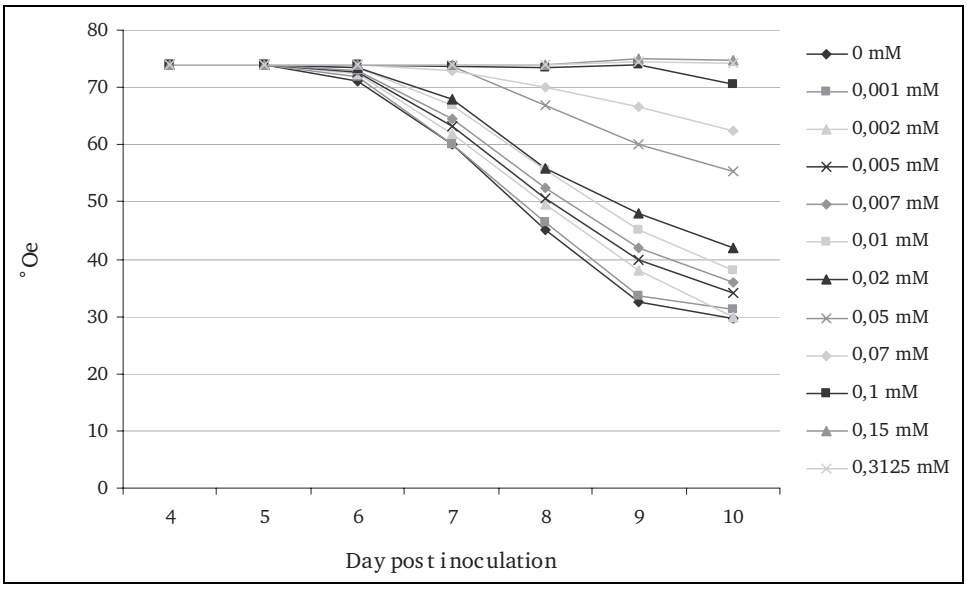


Fig. 3. Effect of glufosinate ammonium (Basta®) on fermentation of grape juice by yeast (*Saccharomyces cerevisiae*). Glufosinate ammonium caused a delay of the onset of fermentation in a dose dependent manner based on the determination of must density (°Oe).

destructive disease in many grape growing regions such as North America (Kuo and Hoch 1996) and Europe (Mohr 2005) when climatic conditions are favourable. Infection of clusters can drastically reduce yield and quality. Ascospores are considered the principal form of primary inoculum but conidia are also produced during the growing season, leading to a mummification of infected berries. Application of fungicides is the primary tactic used to control black rot in most commercial vineyards, even though some fungicides don't seem to provide sufficient control (Hoffman and Wilcox 2003). Interestingly, mycelial growth of *G. bidwellii* was drastically reduced when exposed to low levels of glufosinate ammonium, suggesting an inhibitory effect of this herbicide in the field. This may also be the case for *P. expansum*, the causal agent of green mould on grape, even though vegetative growth was only slightly affected. However, spore production was nearly inhibited on agar plates containing glufosinate ammonium. *P. expansum* is not controlled by chemical treatments, since it occurs late in the growing season when berries mature. The fungus is rarely able to overcome the berry skin but can colonise wounded berries already infected with other parasites. Furthermore, it can spread from infected fruit in contact with healthy ones through the uninjured skin. Unfortunately, wines can be ruined by just a few infected berries that cause wines with mouldy odours and flavours, if these berries are not separated from harvest. Since conidia represent the only important source of infection in the field, an inhibition of spore production, as observed in *in vitro* experiments, may prevent the production of a secondary inoculum and thus an outburst of this disease. The inhibitory effect of glufosinate ammonium on spore production could be observed up to a 500fold dilution of a solution normally applied to weeds. Such low concentrations may occur on berries and left grape mummies if herbicides containing glufosinate ammonium would be sprayed late in the season. Unfortunately, residues on berries may also interfere with fermentation processes, since the yeast *S. cerevisiae* seems also to be highly sensitive to glufosinate ammonium. However, since berries are not treated with herbicides directly, and the active compounds degrade with time due to chemical or microbial decomposition, the negative effect of glufosinate ammonium on yeast seems to be insignificant. Fungi frequently stressed with fungicides can evolve fungicide resistance leading to a reduced control efficiency as already reported in case for *B. cinerea* (Latorre et al. 2002). Spray applications using glufosinate ammonium-based herbicides to control weeds during the growing season may also reach leaf litter or berries infected with saprophytic fungi putatively pathogenic to grapevine at the ground and could help to reduce or avoid the built-up of primary inoculum. Hence, appropriate applications of glufosinate ammonium could be helpful in controlling weeds and in inhibiting diseases due to its side effects on pathogenic fungi. In general, several agrochemicals annually applied may have an effect on parasitic or beneficial microorganisms and may thus also effect the plant's health.

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