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Reduction of olive pollen germination when exposed to herbicides *in vitro*

Redução da germinação de pólen de oliveira quando exposto a herbicidas *in vitro*

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Highlights

The pollen of olive trees exhibits sensitivity to glyphosate and 2,4-D.
Glyphosate and 2,4-D completely inhibited pollen germination.
This study underscores herbicide effects on pollen, urging field research.

Abstract

The main objective of this research was to investigate the possibility of pollen germination reduction in 'Arbequina' and 'Koroneiki' olive cultivars bdue to contamination by glyphosate (N-(Phosphonomethyl) glycine) and 2,4-D (2,4-dichlorophenoxyacetic acid).. Underdoses of glyphosate (0.19 and 1.92 g L^{-1}) and 2,4-D (0.16 and 1.61 g L^{-1}) were added to the culture medium for pollen grain germination. A control treatment, without herbicide addition, was also included in the trial. The experimental design was completely randomized, with three replications. Each plate was a replication and each one consisted of 100 pollen grains were counted after incubation. Pollen grains were considered germinated when their tube length was at least twice of their diameter. The results inferred that there were reductions in germination of 44% and 38.2% for Arbequina and Koroneiki, respectively, when they were subjected to a underdose of 0.19 g L^{-1} of glyphosate. Total inviability for both cultivars, that is 100% non-germinated pollen, occurred at the underdose of 1.92 g L^{-1} of glyphosate and for both underdoses of 2,4-D (0.16 and 1.61 g L^{-1}). The results suggest that there was a high sensitivity of olive pollen to extremely low doses of glyphosate and 2,4-D *in vitro.* It represents a concern about olive groves`productivity due to the recurrence of herbicide drift during the olive flowering period.

Key words: Pesticide. Pollination. *Olea europaea* L.

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Resumo

O principal objetivo desta pesquisa foi investigar se a contaminação por glifosato (N-(fosfonometil) glicina) e 2,4-D (ácido 2,4-diclorofenoxiacético) reduz a porcentagem de germinação de grãos de pólen das cultivares de oliveira 'Arbequina' e 'Koroneiki'. Subdoses de glifosato (0,19 e 1,92 g L^{-1}) e 2,4-D (0,16 e 1,61 g L^{-1}) foram adicionadas ao meio de cultura para germinação de pólen. Um tratamento controle, sem adição de herbicida, também foi incluído no experimento. O delineamento experimental foi inteiramente casualizado, com três repetições. Cada placa constituiu uma repetição, e em cada uma foram contados 100 grãos de pólen após a incubação. Considerou-se germinado o grão de pólen cujo comprimento do tubo polínico era o dobro do seu diâmetro inicial. Os resultados indicaram reduções na germinação de 44% e 38.2% para Arbequina e Koroneiki, respectivamente, quando expostas à subdose de 0,19 g L^{-1} de glifosato. A inviabilidade total para ambas as cultivares, com 100% de pólen não germinado, ocorreu na subdose de 1,92 g L^{-1} de glifosato e nas duas subdoses de 2,4-D (0,16 e 1,61 g L^{-1}). Os resultados sugerem uma alta sensibilidade do pólen de oliveira a doses extremamente baixas de glifosato e 2,4-D *in vitro,* o que representa uma preocupação para a produtividade dos olivais devido à recorrência da deriva de herbicidas, durante o período de floração das oliveiras.

Palavras-chave: Pesticida. Polinização. *Olea europaea* L.

The pesticides' drift, which corresponds to the displacement of these chemicals outside the application area (Bish et al., 2021), results in environmental contamination and imposes toxicological risks to humans and animals (Zaller et al., 2022). Additionally, it impairs the development, survival, and productivity of sensitive plant species, including fruit-bearing plants (Dintelmann et al., 2020).

The contamination by pesticides, including herbicides, can compromise pollen viability (Cappellari et al., 2024; Golt & Wood, 2021) which refers to germination and development of pollen tube, essential for male gametophytes' transportation (Calabrese & Agathokleous, 2021) a crucial aspect for fertilization and reproduction of pollination-dependent species, such as olive trees (*Olea europaea*).

Despite the highlighted importance of herbicide drift, there is a substantial gap in scientific research regarding the direct impacts that this drift exerts on pollen viability in fruit species. This gap assumes critical relevance, especially in the context of olive trees, whose flowering period is during spring (Aguilera et al., 2015) and coincides with the routine application of herbicides in agricultural regions dedicated to grain cultivation, such as soybean.

Considering the lack of data regarding the sensitivity of olive pollen to herbicides, the in vitro evaluation of pollen response to different herbicides provides a relevant preliminary analysis. This approach may serve as a foundation for subsequent field studies, wherein drift simulations can be conducted on both plants and pollen to assess impacts on reproductive processes.

Based on this information, this study aimed to investigate the effects of two commonly used agriculture herbicides, glyphosate and 2,4-D, on *in vitro* pollen viability of two olive cultivars, based on their germination percentage.

The study utilized pollen from Arbequina and Koroneiki cultivars, obtained from a commercial orchard in Pinheiro Machado, Rio Grande do Sul State, Brazil (UTM 31.5607715S; 53.511726W). Inflorescences were collected at stage 59 of BBCH scale (Sanz-Cortés et al., 2002) and kept in polypropylene boxes at room temperature during 24h00 for anthesis and pollen release.

Pollen germination was evaluated on a culture medium, adapted from Silva et al. (2016), containing 8 g L^{-1} agar, 100 g L^{-1} sucrose, and 200 mg L^{-1} boric acid. The experiment incorporated underdoses of commercial herbicides into the culture medium and included a control treatment without herbicide. The underdoses used were 0.16 and 1.61 g L^{-1} of 2,4-D and 0.19 and 1.92 g L^{-1} of glyphosate, respectively 1,000-fold and 10,000-fold less than the commercial dose. The experimental design was completely randomized, with three replications, and each plot was represented by one plate where 100 pollen grains were counted after incubation.

After preparing the culture medium, 20 mL were transferred to Petri dishes, where pollen grains were evenly distributed. Then, the plates were incubated at 26 ºC for 24 hours in a BOD chamber. Pollen grain analysis was performed using a Discovery V20 Zeiss stereomicroscope with AxioVision software. In each plate, 100 pollen grains were counted by rotating the plate clockwise, avoiding double counting. Germination was defined as a formation of a pollen tube with a length at least twice the initial pollen grain diameter (Silva et al., 2016).

The addition of herbicides to the culture medium had a significant impact on the germination percentage. In the control group, without herbicide, a total germination of 25% for Arbequina and 35.6% for Koroneiki was observed (Table 1). The use of glyphosate, at a underdose of 0.19 g L^{-1}, resulted in a reduction in total germination of 44% for Arbequina and 38.2% for Koroneiki. Underdoses of 1.92 g L^{-1} of glyphosate and 0.16 and 1.61 g L^{-1} of 2,4-D completely inhibited pollen grain germination on both cultivars (figure 1).

Table 1
Pollen grain germination percentage of Arbequina and Koroneiki cultivars under glyphosate and 2,4-D *in vitro*

Herbicide	Underdoses (g L^{-1})	Germination (%)	
		Arbequina	Koroneiki
Control treatment	0	25.00 a	35.60 a
Glyphosate	0.19	14.00 b	22.00 b
	1.92	0.00	0.00
2,4-D	0.16	0.00	0.00
	1.61	0.00	0.00
cv %		24.28	

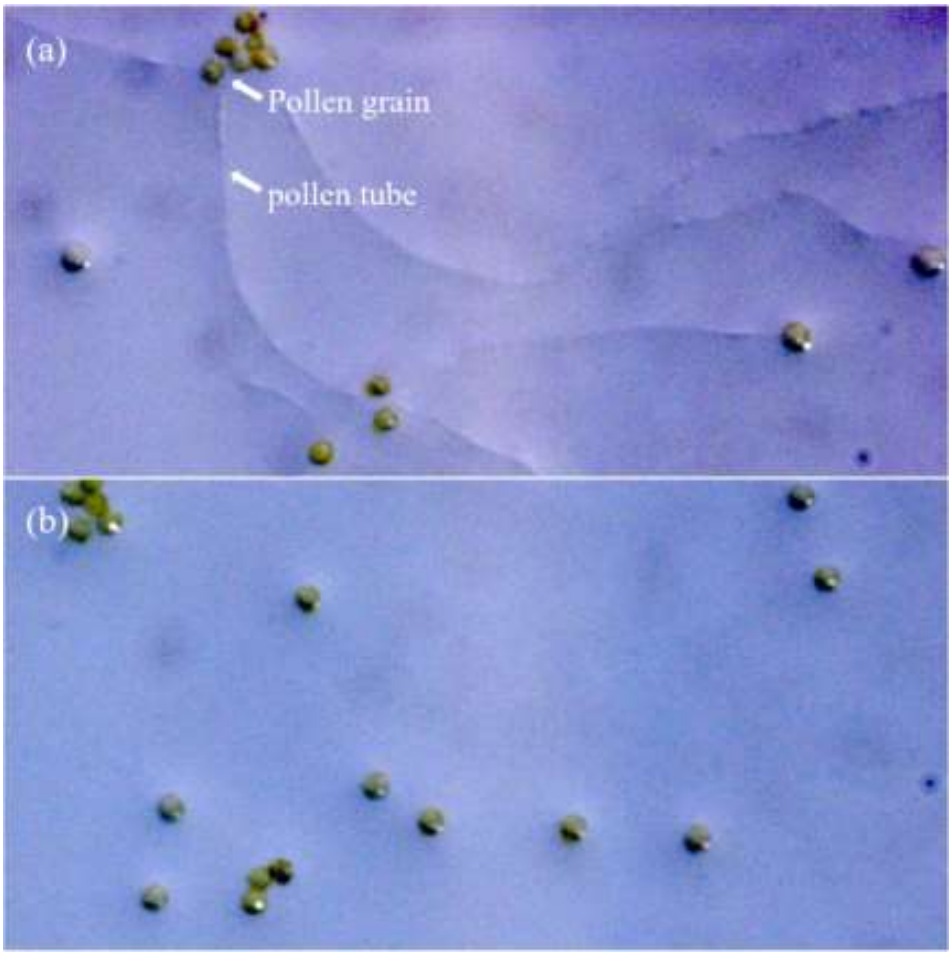

Figure 1. Pollen grains of the Koroneiki olive in culture medium without herbicide, showing the development of pollen tubes (a), and with the herbicide 2,4-D, where no pollen tube development is observed (b).

The decrease in pollen grain viability due to glyphosate has been observed in other species (Golt & Wood, 2021), including glyphosate-resistant corn (Thomas et al., 2004). However, the influence of glyphosate on olive pollen grain viability had not yet been researched.

Glyphosate inhibits enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is essential in shikimic acid pathway for biosynthesis of aromatic amino acids (Duke, 2021). This occurs because glyphosate competes with the substrate phosphoenolpyruvate (PEP), forming a ternary complex with the second substrate of EPSPS, shikimate-3-phosphate (S3P), and resulting in the inhibition of chorismate production, a precursor of aromatic amino acids (Light et al., 2016).

The highest concentration of glyphosate used in this study (1.92 g L^{-1}) did not allow pollen grain germination, whereas in other species, such as Soligado canadenses, germination is inhibited from a substantially higher underdose (10 g L^{-1}) (Guo et al., 2009). This result indicates that olive pollen is sensitive to glyphosate, the most widely used herbicide in global agriculture, which is also employed in weed management in olive groves (Peragón & Amores-Escobar, 2018).

The herbicide 2,4-D is known to mimic the function of plants' endogenous auxin, indoleacetic acid (IAA), and it can act as a growth regulator at reduced concentrations (Grossmann, 2010). In this context, the lack of germination observed at sublethal concentrations of 2,4-D (0.16 and 1.61 g L^{-1}) may be attributed to the sensitivity of olive pollen and the herbicide intrinsic toxicity.

Although it shares similarities to auxins, 2,4-D demonstrates remarkable stability (Song, 2014), resulting in supra-physiological concentrations capable of disrupting plant mechanisms. It causes damage to membrane integrity and triggers adverse physiological events (Grossmann, 2010).

The complete inhibition of germination by 2,4-D at low sub-doses highlights its high toxicity to 'Arbequina' and 'Koroneiki' pollen, whereas glyphosate only showed inhibitory effects at higher underdoses, suggesting a lower toxicity profile when compared to 2,4-D. These results indicate the need for field research to assess the effects of the herbicide on pollen when sprayed on the tree canopy or branches, simulating real drift conditions.

Pollen of 'Arbequina' and 'Koroneiki' olive is highly sensitive to herbicides glyphosate and 2,4-D under *in vitro* conditions. Glyphosate at the underdose of 0.19 g L^{-1} reduced germination rate by 44% and 38.2%, respectively. At higher underdoses of glyphosate used in this study (1.92 g L^{-1}) and 2,4-D (0.16 and 1.61 g L^{-1}), germination was completely inhibited.

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