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Predicting biphasic responses in binary mixtures: Pelargonic acid versus glyphosate

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HIGHLIGHTS

- Hormesis in binary mixtures of two commercial herbicides was investigated *in vitro*.
- Low doses of both herbicides and their mixtures stimulated plant growth up to 51%.
- A linear y_{max} model assuming additivity served as reference for low-dose responses.
- Maximum stimulation in mixture either followed a linear trend or varied atypically.
- A model to predict deviations from additive hormetic mixture responses is proposed.

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ABSTRACT

Predicting hormesis in mixtures is challenging, but essential considering that chemical exposures often occur in mixtures and at low doses. This study investigated mixture effects with two herbicides prone to induce hormesis and to interact, namely pelargonic acid versus glyphosate. Five independent mixture experiments were conducted *in vitro* to assess effects on root growth of lettuce. Mixture effects on the dose were analyzed using classical joint-action models in terms of deviation from the reference model of concentration addition. For effects on the hormetic magnitude (y_{max}), a linear reference model was utilized.

Hormesis was inconsistent across rays, so that effects on inhibitory doses and y_{max} could be evaluated, but not effects on hormetic doses. Mixture effects on the dose were additive at lower doses changing to strong high-dose synergism. Mixture effects on y_{max} followed a linear change with mixture ratio or significantly deviated from linearity with a one-sided trend across rays in two experiments. The trend was antipodal between experiments, but well described by a curved y_{max} model based on single dose-response relationships. Atypical y_{max} deviations were associated with strong synergism at ED_{50}, suggesting that the linearity model applies for chemicals showing no/minor interaction at ED_{50}, while for strongly interacting chemicals y_{max} predictions seem more critical.

The study unambiguously proved synergism on the dose for pelargonic acid versus glyphosate and indicated an impact of these joint effects on y_{max}. The study confirms the predictability of hormesis in mixtures and provides a further methodological step towards an incorporation of hormesis into mixture-toxicity evaluations.

1. Introduction

Biphasic or else hormetic dose responses signify stimulatory effects on one or several organism traits by low-doses of stressors including toxins such as herbicides that change to inhibitory effects at higher doses. This phenomenon of hormesis is becoming a more and more widely accepted occurrence in many toxicological disciplines (Calabrese, 2008a). Dose-response relationships allowing for hormesis can meanwhile be well described with mathematical models that facilitate establishing the significance of stimulation and to deduce several quantitative features of hormesis, be it a single or multiple toxins impact on an organism (*e.g.*, Belz et al.,

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2008; Ohlsson et al., 2010; Bain and Kumar, 2014). The issue of hormesis induced by multiple toxins is highly relevant for many toxicological disciplines as organisms are mostly exposed to more than one toxin at a time and often to low doses of these mixtures (*e.g.,* environmental pollutants, pesticide residues, or allelochemicals). While joint effects in toxin mixtures can be straightforwardly modelled in the inhibitory dose range (Sørensen et al., 2007; Ritz and Streibig, 2014), the evaluation of joint effects in the stimulatory dose range is still a challenging task. The challenge includes two aspects: *First*, prediction of the effective doses characterizing hormesis. This mainly includes the dose *M* leading to maximum stimulation and the *LDS* (limited dose for stimulation) or *ZEP* (zero equivalent point), *i.e.*, the dose where stimulation has vanished. *Second,* prediction of the magnitude of hormesis. This includes the maximum stimulatory response E(y) at *M* or y_{max}.

The *first aspect* has been previously addressed studying binary or ternary mixtures showing hormesis by adopting generally accepted joint-action models that have been developed for monotonic mixtures (*e.g.*, Belz et al., 2008; Ohlsson et al., 2010; Ge et al., 2011; Zhang et al., 2014). The majority of these studies showed that hormetic doses can equally well be predicted by traditional reference models such as *concentration addition* (CA) in case of additivity or the *Hewlett* or the *Vølund* isobole models in case of synergism/antagonism. All of these models predict mixture effects based on the dose-response relationships of the single compounds. Hence, if all mixture partners show hormesis, the hormetic dose range can be well predicted using available joint-action models (Belz et al., 2008; Ohlsson et al., 2010). When it comes to the *second aspect* and, thus, to the prediction of y_{max} in mixtures, the situation is not so easy. Currently, there are no mechanistic models available that could be adopted nor is there a generally accepted model available to predict y_{max} in mixtures (Ohlsson et al., 2010). Based on empirical observations in binary mixtures with both or one of the mixture partners showing hormesis, Belz et al. (2008) found the majority of their datasets to follow a linear change of y_{max} values with the mixture ratio. The deduced *'linearity model'* for y_{max} predictions in mixtures is thus similarly based on the dose-response relationships of the single compounds or more precisely their y_{max} values. The model also proved reasonable to predict mixture effects on y_{max} in a study of Ohlsson et al. (2010) and is in line with the *'hormetic mixture concept'* proposed by Calabrese (2008a,b). This concept implies that synergism/antagonism in a hormetic framework relates primarily to the dose, meaning that hormesis in mixtures is achieved without a marked change in y_{max} at a lower/higher combined dose. The rationale for this assumption is the limited biological plasticity that narrows the maximum stimulatory potential of an organism trait to a typical boost of 30–60% above controls (Calabrese, 2008a,b). The hormetic mixture concept is thus different from our understanding of mixture effects in the toxic response zone where an interaction is always changing the magnitude of the toxic response (Calabrese, 2008a,b). Since little consideration is still given to the prediction of hormesis in mixtures, it is yet unsettled if the linearity model is generally applicable as a reference model for y_{max} and if atypical deviations in y_{max} exceeding a typical boost are really against expectation as proposed by the hormetic mixture concept.

In both previous studies predicting y_{max} in mixtures, the linearity model well described the data while mostly no interaction occurred on the dose (Belz et al., 2008; Ohlsson et al., 2010). Atypical deviations from the linearity model were only observed for *Lemna minor* L. exposed to biphasic mixtures of two herbicides interacting strongly antagonistic on the dose (Belz et al., 2008). Based on this, it seems of utmost importance to study the validity of the linearity model and, thus, also the hormetic mixture concept in case of chemical interaction. This was currently done by selecting mixture compounds that are prone to interact and to induce hormesis, namely the natural herbicidal agent pelargonic acid (PA) and the most used synthetic herbicide glyphosate (GLY). PA is claimed to synergize the phytotoxicity of certain postemergence herbicides like GLY, which is why mixtures of PA and GLY are sold as ready-to-use weed control products or are recommended as tank mixtures (Pline et al., 2000; Chachalis and Reddy, 2004; Wehtje et al., 2009). The mixture is thus of practical relevance for weed control, although no mixture study yet convincingly supported the claimed synergism and additivity or antagonism has thus far been reported, especially at mixture ratios equalling those of ready-to-use weed control products (*e.g.*, Pline et al., 2000; Chachalis and Reddy, 2004; Wehtje et al., 2009). Nevertheless, it is evident that both herbicides can interact and GLY is especially well known to induce hormesis in several plant traits and species (Cedergreen, 2008a,b; Velini et al., 2008). Furthermore, a previous study indicated unexpected hormesis in mixtures of PA versus GLY (Belz and Leberle, 2012). Therefore, a comprehensive mixture study allowing for a joint-action evaluation of PA versus GLY at various mixture ratios and at various dose levels was conducted under the following hypotheses: (1) PA synergizes the activity of GLY so that mixture effects on the dose do not follow the reference model of CA, but can be predicted by the curved isobole model of Hewlett at various dose levels; and (2) interactions on the dose are associated with atypical deviations of y_{max} from the linearity model that exceed the typical range of maximum stimulatory responses, thus contradicting the proposed hormetic mixture concept. In order to evaluate this second hypothesis, a statistical procedure to verify the significance and degree of y_{max} deviations was established and a curved model to predict atypical y_{max} deviations is proposed.

Experiments comprised *in vitro* dose-response germination bioassays with *Lactuca sativa* L. (lettuce) as test plant species exposed in a classical fixed-ratio ray-design to the two single herbicides PA and GLY or their mixtures. Dose-dependent growth responses were evaluated for root elongation, as this endpoint has shown high stimulatory plasticity for lettuce. Since hormetic responses as well as chemical interactions were shown to be susceptible to between-experiment variation (*e.g.,* Cedergreen et al., 2007a; Belz and Piepho, 2013), the entire mixture experiment was repeated five times.

2. Material and methods

2.1. Herbicides

The herbicides were used as commercially formulated products, namely 'Bayer Garten 3 Stunden Bio-Unkrautfrei' with 186.7 g a.i./l pelargonic acid (PA) (Bayer CropScience AG) and 'Glyfos Supreme' with 450 g a.i./l glyphosate (GLY) (Cheminova Deutschland GmbH). We preferred to work with formulated products since in practice, both compounds interact in formulated ready-to-use products (*e.g.*, Roundup Speed, Monsanto Agrar Deutschland GmbH) or in the form of tank-mixtures of the single commercial herbicides (*e.g.*, Pline et al., 2000).

2.2. Lettuce bioassay

The test method has been used and published previously (*e.g.*, Belz et al., 2008; Belz and Piepho, 2013). Briefly, the assay comprised dose-response germination experiments with *L. sativa* cv. Maikönig (Blauetikett-Bornträger GmbH, Germany) as test plant and root elongation as endpoint. The experiment was done in 6-well cell culture plates (Cellstar, greiner bio-one). Each well was prepared with one layer of filter paper (Ø 34 mm, MN 615, Macherey-Nagel) and by adding five lettuce seeds. Thereafter,