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Effects of herbicide and nitrogen fertilizer on non-target plant reproduction and indirect effects on pollination in *Tanacetum vulgare* (Asteraceae)

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ABSTRACT

Herbicides and nitrogen fertilizers are widely used in intensively grown agricultural areas. Non-target plants growing in habitats adjacent to conventional fields may be exposed to herbicides by spray drift and misplacement of nitrogen fertilizers. Whereas sub-lethal effects of herbicides have previously been documented, combined effects of nitrogen and sub-lethal herbicide exposure are less well known. Moreover, indirect effects on pollination and fruit set following effects of agrochemicals on flowering remain largely unexplored. In the current study, we investigate combined effects of herbicide and nitrogen fertilizer on reproductive features of *Tanacetum vulgare* (Asteraceae). The study was carried out in an experimental set-up, in which plots of 7×7 m were treated with one of six treatments: four levels of the herbicide glyphosate (0%, 1%, 5%, and 25% of label rate of 1440 g a.i. ha^{-1}) without nitrogen in addition to two levels of herbicide (0% and 25%) with added nitrogen (100 kg N ha^{-1} $year^{-1}$). The set-up had a randomized full-factorial design with 10 replicates of each treatment. We monitored floral density per plot every fortnight during flowering, stem height at flowering, diameter of capitula, visitation rate and diversity of flower-visitors, in addition to weight per seed and total seed weight per seed head. Glyphosate, nitrogen and their interaction affected plant reproductive features, in particular, floral density and flowering phenology. Glyphosate negatively affected floral density, and flowering was severely delayed by glyphosate application (10.5 days delay per 100 g a.i. ha^{-1} $year^{-1}$). Although nitrogen partly mitigated reduction in floral abundance, the delay in flowering was amplified when nitrogen was added. Effects of glyphosate on flowering were mirrored in reduced flower visitation by insects and reduced seed set. These effects occurred even when plants were exposed to glyphosate two months before onset of flowering. Our results show that misplacement of agro-chemicals markedly changes floral abundance and flowering phenology of non-target plants, which may secondarily impact pollination interactions and reproduction of wild plants in agricultural landscapes.

1. Introduction

Wild plants, including weeds in agro-ecosystems, are key components of food chains for associated fauna (insects and birds) and, hence, support biodiversity (Marshall et al., 2003). In Europe, many native plants, including important food plants of flower-visiting insects, have shown marked historical reductions in range and decline in abundances (Sutcliffe and Kay, 2000; Biesmeijer et al., 2006; Carvell et al., 2006). Diversity and biomass of non-target plant communities in semi-natural areas adjacent to conventionally grown fields are affected by drift of agrochemicals, most notably herbicides and nitrogen fertilizers (Kleijn and Snoeijing, 1997). Assessing the potential impact of spray drift from agricultural fields on natural vegetation is of growing concern for pesticide regulation (Carpenter and Boutin, 2010; Boutin et al., 2014; EFSA PPR Panel, 2014).

1.1. Sub-lethal effects of herbicide spray drift exposure

A range of different herbicides, including broad spectrum and selective herbicides, are used in agricultural and non-agricultural areas. In the current study, we investigate the effects of the broad spectrum systemic herbicide glyphosate (*N*-(phosphonomethyl)glycine), the active ingredient of Roundup, which is the most widely used herbicide worldwide (Benbrook, 2016). It is applied in spring for weed control, in mid and late summer prior to harvest for crop desiccation, and for wilting of grass and cover-crops in autumn and early spring. In a cereal crop, the field margin is exposed to herbicides due to direct exposure and unintentional diffusion of herbicide when spraying is conducted at the field edge. The average exposure within one meter of the field edge has been estimated at 39.5% of the field application rate (Schmitz et al., 2013 and references therein). However, actual exposure is highly

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affected by the spraying equipment and weather conditions during spray application, and pesticide spray drift concentrations are mostly in the range of 5% to 25% of the field application rate (Holterman et al., 1997; Weisser et al., 2002). Spray drift exposure mainly results in sub-lethal doses of herbicides. Previous studies investigating effects of spray drift exposure report reductions in diversity (e.g. Kleijn and Snoeijing, 1997; Pedersen et al., 2004; Schmitz et al., 2014a), as well as species-specific alterations in cover (Egan et al., 2014; Strandberg et al., 2017), demographic vital rates (Damgaard et al., 2013), and interspecific competitive interactions (Damgaard et al., 2008; Damgaard et al., 2011, 2014). Furthermore, sub-lethal doses of herbicides have been shown to affect flower, fruit and seed set of herbaceous plants (e.g. Olszyk et al., 2009; Boutin et al., 2014). Several studies have documented reduced floral abundance (Olszyk et al., 2009; Carpenter and Boutin, 2010; Strandberg et al., 2012; Carpenter et al., 2013; Schmitz et al., 2013; Boutin et al., 2014; Schmitz et al., 2014b; Strandberg et al., 2017) and Boutin et al. (2014), in addition, showed significant delay in flowering following herbicide application. Decline in flower abundance and/or shifts in flowering phenology resulting in loss of floral resources is considered as a major factor driving decline of native flower-visiting insects (Potts et al., 2010). Thus, herbicide spray drift exposure of non-target wild plants may have indirect effects on flower-visiting insects and, ultimately, on pollination and seed set. To our knowledge, these indirect effects remain unexplored.

1.2. Effects of misplaced nitrogen fertilizer

In addition to pesticides, field margins are exposed to fertilizer misplacement (Tsiouris and Marshall, 1998). Elevated levels of nitrogen leads to species-poor plant communities dominated by few, fast-growing, competitive species, most notably grasses (Kleijn and Snoeijing, 1997; Kleijn and van der Voort, 1997; Tsiouris and Marshall, 1998; Vickery et al., 2001), which do not provide floral resources for flower-visiting insects (Proctor et al., 1996).

1.3. Combined effects of herbicide and nitrogen

Few studies have investigated long-term and combined effects of exposure to nitrogen fertilizer and herbicide drift on non-target wild plant species, even though these two important drivers often co-occur in semi-natural vegetation adjacent to agricultural fields (but see Damgaard et al., 2011, 2013). In a case study, Schmitz et al. (2013) investigated sub-lethal effects of Atlantis WG (active ingredients mesosulfuron and iodosulfuron), and nitrogen (25% field application rate or 50 kg N ha^{-1} year^{-1}) on *Ranunculus acris* L. Plant density decreased with nitrogen fertilizer application, and flowering intensity was reduced by 85% following exposure to 30% label rate (400 g a.i. ha^{-1}) of the herbicide (Schmitz et al., 2013). In a study of woodland ground flora, six herbaceous plant species were exposed to spray drift concentrations of glyphosate and nitrogen fertilizer at concentrations simulating displacement from the field (Gove et al., 2007). Survival, biomass and fecundity (proportion of flowering plants) were reduced by glyphosate treatment, while nitrogen fertilizer did not alter fecundity of most of the tested species, and no interaction effects between glyphosate and nitrogen were found (Gove et al., 2007).

1.4. Study objectives

The purpose of this study is to investigate the effects of an herbicide, glyphosate, a nitrogen fertilizer and their interaction on reproductive features of potential importance to pollination in *Tanacetum vulgare* L. (Asteraceae). We use a long-term semi-field set-up in which experimentally established grassland plots are treated with spray drift concentrations of glyphosate combined with different levels of nitrogen. We specifically investigate whether glyphosate and/or nitrogen affect (1) floral abundance and phenology, (2) plant morphology (stem height and capitula size), (3) flower visitor activity and diversity and (4) seed set of *Tanacetum vulgare*. The results are discussed in a wider context of effects of the use of agrochemicals on flowering and indirect effects on pollination of non-target wild plants in semi-natural biotopes adjacent to treated fields in intensively farmed agricultural landscapes. These results are highly relevant for regulating the use of herbicides and for conservation of wild flowers and flower-visiting insects.

2. Materials and methods

2.1. Experimental set up

The study was conducted using a sub-set of plots in a long-term semi-field experiment at Ebdrup mark, Denmark (56°20′2“N 10°34′30“E). The experiment was established in 2001 to investigate spray drift levels of herbicides and misplacement of nitrogen fertilizers to habitats adjacent to treated fields. Specifically, the experiment was set up to simulate a scenario of spray drift and misplacement of agrochemicals from a treated cereal field on semi-natural grassland vegetation (http://bios.au.dk/faciliteter/long-term-experimental-plot/). Within the experimental area, plots of 7 × 7 m were treated annually with one of four levels of glyphosate (0, 14.4, 72 and 360 g a.i. ha^{-1} year^{-1}, equivalent to 0, 1, 5 and 25% of label rate of 1440 g a.i. ha^{-1} year^{-1}) combined with three levels of inorganic nitrogen (0, 25 or 100 kg N ha^{-1} year^{-1}). Roundup Bio®, the commercial formulation of glyphosate (National Pesticide Information Center, 2017) was used for herbicide application. The herbicide concentrations correspond to field relevant doses of spray drift (see Section 1.1). The recommended rate of nitrogen fertilizer application for cereal crops in Denmark is 180–236 kg N ha^{-1} (SEGES, 2017). As fertilizer misplacement within one meter of the treated field has been estimated at 25–50% of the field application rate (Tsiouris and Marshall, 1998), the 100 kg N ha^{-1} year^{-1} treatment in the experiment may be considered a worst case scenario of fertilizer misplacement. Plots were assigned randomly to treatments within each of 10 blocks in a full factorial design (Appendix A). All plots were fertilized with phosphorus (53 kg ha^{-1}), potassium (141 kg ha^{-1}), sulphur (50 kg ha^{-1}) and copper (0.7 kg ha^{-1}) every year. Plots were not watered, and except for removal of tree saplings once a year, the vegetation was not cut (Pedersen et al., 2004). For further details of the experimental set-up, see Appendix A.

The year of the current study, fertilizers were applied the 30 April 2013 and glyphosate was applied on the 23 May 2013. On the day of glyphosate treatment, the weather was sunny, wind speed was 0–3 m s^{-1}, the temperature 12–15 °C, and there was no rain, nor did it rain during the days following treatments. The application was carried out by equipment adapted for experimental application. The beam was 3 m with 0.5 m between the nozzles (Lurmark Lo-drift LD 015 Green nozzles with a pressure of 2.0 bar).

In the current study, we measured plant reproductive features which could affect interactions with insect pollinators (see below). Due to time constraints, we collected data only in plots representing six of the 12 different treatments in the semi-field experiment: four levels of herbicide concentrations combined with no nitrogen application (0 kg N ha^{-1} year^{-1}), in addition to 0 (no herbicide) and 360 g a.i. ha^{-1} year^{-1} (highest herbicide concentration) combined with a high level of nitrogen fertilizer (100 kg N ha^{-1} year^{-1}). Data were collected from June to November 2013

2.2. Study species

Tanacetum vulgare, Tansy, is an herbaceous perennial plant which occurs in semi-natural vegetation in agricultural landscapes of Northern Europe. It grows in field margins, road sides, waste areas and grasslands and, hence, commonly occurs in areas adjacent to agricultural fields. The plant is clonal and grows as clusters of erect stems of 30–150 cm,