On the coexistence mechanisms of a perennial grass with an allelopathic shrub in a semiarid rangeland
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ABSTRACT: An ecophysiological study was conducted on allelopathic effects of a shrub (Artemisia khorassanica) on a perennial grass (Bromus kopetdaghenisis), in Baharkish rangelands, Quchan, Iran. Based on field visits (March – October 2011), Bromus started and terminated its yearly growth respectively one and three months sooner than Artemisia. Water soluble extracts (3\% weight/Volume) were taken from Artemisia shoots, at the beginning of the growth season, full vegetative and flowering stages. In a glasshouse experiment, the Artemisia extracts were applied on Bromus seedlings at early vegetative, full vegetative and flowering stages. Artemisia extracts reduced photosynthetic pigment concentration but increased oxidative stress indices in Bromus shoots. Both the allelopathic effects by Artemisia and the adaptive responses from Bromus were reduced by the growth season. Therefore, temporal differences in the phenology, temporal shifts in the allelopathic effects and responses, and Bromus acclimations to mediate the allelopathic stress, may be the reasons for their natural coexistence.

Keywords: Allelopathy, H\textsubscript{2}O\textsubscript{2}, Lipid peroxidation, Peroxidase enzyme activity, Phenology, Pigment concentrations

INTRODUCTION

Understanding the coexistence mechanisms between plants will be useful for maintenance of species diversity and management of degraded ecosystems. Plants may affect other plants growing in their vicinity in a stimulatory or inhibitory manner, through released biologically active compounds often termed as allelopathics, allelocompounds or allelochemicals. This phenomenon is termed as allelopathy, receiving an increased attention recently and is considered to be applied in practice for weeds and pest managements (Prasanta et al., 2003). It can affect many aspects of plant ecology including the dominance, succession, diversity and structure of plant community (Molisch, 1937).

Several Asteraceae species are known for having allelopathic compounds, which may reduce seed germination and seedling emergence of other plants (Al-Watban and Salama, 2012). Artemisia is a widely distributed genus from Asteraceae. It includes 200 to 400 species in different biomes, which are predominantly growing in the northern hemisphere (Pirzad et al., 2010). In Iran, 34 species of Artemisia are reported, some of them being endemic (Motaghinia, 2002). Some Artemisia species e.g., A. herba-alba, A. annua, A. teridentata and A. princes produce a large variety of secondary metabolites such as flavonoids and comarines, which may cause allelopathic effects on neighboring plants (Lydon et al., 1997; Groves and Anderson, 1981; Nabeel et al., 2006; Deef and El-Fattah, 2008). Leaf extracts of A. dubia caused an inhibitory effect on the germination and growth of barnyard grass (Pudel et al., 2005). Aqueous extract from leaf and inflorescence of A. monosperma decreased seed germination and growth of Lasiurus scindicus, Pennisetum divsum, Scrophularia hypericifolia and Pennisetum boisseri (Assaeed, 2003). Aqueous extract of A. princes decreased germination, growth and chlorophyll contents in Triticum aestivum (Deef and El-Fattah, 2008). Al-Watban and Salama (2012) showed that protease and amylase activity and total soluable sugar of growing Phaseolus vulgaris L. were decreased by concentration of aqueous extract of A. monosperma. The volatile oil of Artemisia ordosica resulted in inhibition the growth and photosynthetic activity of Palmmelllococcus miniatus (Yang et al., 2012).

Artemisia khorassanica (hereafter called Artemisia) is a semi-shrub species, widely distributed in the northeast Iran. It produces chemicals such as essential oils (camphor, 1, 8-cineol, avanone and isogeraniol) and phenolic compounds (coumarins, flavonoids) (Kil et al., 2000; Ghorbani-Ghouzhdi et al., 2008), which can serve as
allelopathic agents in plants (Razavi, 2012). Previous studies in Baharkish rangelands, Quchan, Iran (Jankju et al., 2010) showed that a perennial grass, Bromus kopetdaghensis (hereafter called Bromus) can naturally establish within the canopy of Artemisia. Nevertheless, Behdad et al., (2011) in a laboratory experiment found allelopathic effects of Artemisia on seed germination and seedling growth of Bromus. Therefore this experiment was aimed to study the physiological ecology aspects of Artemisia allelopathy on Bromus seedlings, during different phenological stages in a single growth season.

Allelopathic effects may be affected by the environmental factors and/or its intensity may change by time. For example, Maghamnia et al., (2010) found that in early times of growth season Artemisia had facilitative effect for soil water on Bromus, but during the summer drought the relationship was changed to the negative effects. Similar to these results, Berger (2007) has shown that allochemicals produced by plants vary depending on their growth stages and habitats. Also, Bagheri and Mohammadi (2010) reported that allelopathic potential of Artemisia sieberi varied as its secondary metabolites changed under three rangeland utilization rates (i.e. heavy, moderate and no grazing). Accordingly in this experiment, we hypothesized the temporal shifts in importance of positive and negative interactions between Artemisia on Bromus may lead to their coexistence under the natural field conditions.

MATERIAL AND METHODS

Sampling site

The study site was in Baharkish, Quchan, northeast of Iran (58° 09′ – 58° 50′ E and 36° 33′ – 36° 33′ N), with the latitude varying from 1500 – 2700 m.a.s.l. The climate of this region is semi-arid cold with an average annual precipitation of 365 mm (average of 15 years). Total vegetation cover was about 30% which was mainly dominated by shrub species such as Acantholymon prostegium Czernjak, Astragalus meshedensis, Artemisia khorassanica Podl., A. aucheri Boiss with some herbaceous species and perennial grasses such as Stipa barbata Dest., Agropyron trichophorum (Link) K. Richt., Festuca ovina L. and Bromus kopetdaghensis Drobov., which were mainly growing under canopy of the shrubs. The site was under intense livestock grazing during June-August.

Phenological study

Major phenological growth stages of Bromus and Artemisia were recorded by doing two-weekly field visits, during March – October 2011. For this, 10 individual plants were randomly selected and marked for both species. The major phenological growth stages studied were: beginning of seasonal growth (first leaves), early stems (for Bromus), full vegetative, early and late flowering, early and late seed shedding, and leaf mortality and shedding. There were small differences between the individuals of each species, but the new phenological stage was recorded when on the majority of plants (>65%) reached to the same stage.

Field sampling

Current year shoot growth of Artemisia was sampled, three times (early May, Late June and Early September 2011). At each sampling time, three 1 kg combined samples (from 4-5 individual plants) were taken. The samples were air dried and then grinded with pestle and motel, in the laboratory.

Treatments and experimental design

A glasshouse experiment (ambient temperature and 16 hrs light times) was conducted in the department of Botany, Ferdowsi University of Mashhad, Iran. 300 Bromus seeds were sown in seed trays. Bromus seedlings were transplanted into 2 kg pots, after two weeks. The experiment contained 12 treatments, which was a factorial combination of allelopathic treatments (4 extract types) and Bromus growth stages (3 phenological stages) in a completely randomized design. Allelopathic treatments were water soluble extracts (3% W/V) that had been taken from Artemisia shoots (i.e. current year stems and leaves), at the beginning of growth, full vegetative or flowering stage; plus a control (distilled tap water) treatment. Bromus growth stages were: early vegetative, full vegetative and flowering. Each treatment was repeated 3 times; hence the total experimental units were 36. Data were analyzed by a two-way ANOVA, using SPSS16 (SPSS Inc., Chicago, USA.).

Plant harvests

Bromus individuals were harvested 14 days after being treated by Artemisia extracts. This time was 42, 64 and 132 days after transplanting Bromus seedlings into pots, for the first, second and third resource application times respectively. The fresh and dry weights of Bromus root and shoots were measured in each harvest. For measuring dry matters, plant samples were oven dried for 24 hours at 70°C.
Pigment concentrations (Chlorophyll a, b, T and Carotenoids)

For measuring pigment concentrations, 25 mg of Bromus shoots (those treated by allelopathic extracts) were grinded and then dissolved in 2 ml acetone 80% (w/v), for about 10 min. The samples were centrifugated at 3,000 rpm for 5 min and supernatants absorption were measured at A470, A646, A663 nm to estimate pigment concentrations according to an empirical formula proposed by Lichtenthaler and Wellburn (1983).

\[
\begin{align*}
A646(2/81) - A663(12/21) &= \text{Chla concentration (mg/ml)} \\
A663(5/03) - A646(20/13) &= \text{Chlb concentration (mg/ml)} \\
(1000A470-3.27\times \text{Chla}) - (104\times \text{Chlb})/227 &= \text{Carotenoids concentration (mg/ml)} \\
\text{ChlT} &= \text{Chla} + \text{Chlb}
\end{align*}
\]

Lipid peroxidation (Malondialdehyde measurement)

Malondialdehyde (MDA) content was measured in treated and control leaves of Bromus as described by Zhao et al., (1994). For this, 0.1 g of leaf samples was homogenized in 5 ml of 10% trichloroacetic acid (TCA) with a chilled mortar and pestle and then centrifugated at 4,000 rpm for 10 min. Then, 2 ml of supernatant was mixed with 2 ml solution containing 0.6% thiobarbituric acid (TBA) in 10% TCA. The mixture was heated in a boiling bath for 15 min, quickly cooled and then centrifugated at 4,000 rpm for 10 min. Absorbance of the supernatant was determined at 532 nm and 600 nm. The MDA concentration was calculated after subtracting nonspecific absorbance at 600 nm using the extinction coefficient of 155 mM cm$^{-1}$.

Hydrogen peroxide ($\text{H}_2\text{O}_2$)

$\text{H}_2\text{O}_2$ content was determined according to Velikova et al., (2000). Bromus fresh leaves (0.5g) were homogenized with 0.5 ml of 0.1% trichloroacetic acid (TCA) in an ice bath. The homogenate was centrifugated at 4000 rpm for 10 min and 0.5 ml supernatant was mixed with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 10 ml of 1M KI. The absorbance of the mixture was read at 390 nm. The content of $\text{H}_2\text{O}_2$ was calibrated based on the standard curve and measured by using fresh leaf weights.

Peroxidase enzyme (POD) activity

For measuring peroxidase enzyme (POD) activity, 2ml of acetate buffer (0.2M, PH=5), 0.2 ml H2O2 (3%) and 0.1ml of dissolved benzidine (0.02M) in methanol (50%) were mixed in ice bath. Then 0.1ml of leave enzyme extracts were added to this mixture. Enzyme extracts were made from 100-120 mg of the fresh material in 0.1M phosphate buffer (pH 7.0) in an ice bath. The extracts were centrifugated at 5000 rpm for 15 min. Absorbance of the extracts were determined at 530 nm, 3 minutes intervals. The changes in enzyme activity were recorded every 30 seconds. Curve of absorbance changes was plotted. Finally, specific enzyme activity was calculated based on changes in absorbance units per minutes for each milligram of protein (Hoyl, 1972).

RESULTS

Phenology of Bromus and Artemisia

There were time differences between the major phenological stages of Artemisia and Bromus, under field conditions (Table 1). Bromus started its vegetative growth one month sooner than Artemisia did. The flowering stage was also 40 days sooner for Bromus than Artemisia. Bromus ended its seasonal growth by the early August, while Artemisia continued until three months later (early November). The mature individuals of Bromus were dormant during October – March. However, at the same period, some of its seed bank germinated and seedlings were doing a slow growth.

Bromus dry mass

The allelopathic compounds of Artemisia extracts reduced the total dry weight of Bromus seedlings, but the differences were not statistically significant and did not follow a specific pattern with time (hence the data not presented). In the control treatment (i.e. no allelopathy), Root:shoot ratio (RShR) of Bromus was not affected by plant age (Figure 1). Artemisia extracts led to a higher RShR for Bromus seedlings, under the allelopathic treatments as compared with the control. Nevertheless, the allelopathic was compromised by the plant age; young shoots of Artemisia showed the highest allelopathic effects, but the effect was decreased as the shrub reached to the flowering stage (Figure 1, compare line for early vegetative with that of flowering).
Chlorophyll contents
Chlorophyll a in Bromus leaves was highest at the early vegetative growth (day 42) and gradually decreased by the flowering stage (day 132) (Figure 2). Such reduction was found for Bromus seedlings that were growing both under the control and the allelopathic treatments, but application of Artemisia extracts intensified reduction of Chlorophyll a. Moreover, the allelopathic effects of Artemisia were dependent on its phenological stages. The extract taken at the early vegetative growth of Artemisia caused the greatest reduction in chlorophyll a and the effect was linearly reduced towards at the full vegetative and flowering stages (Figure 2). Effects of phenological stage and Artemisia extracts on total chlorophyll and chlorophyll b were similar to those described for chlorophyll a (data not shown).

Carotenoids
Carotenoids content was increased in Bromus leaves, as it was aging from early towards the flowering stage (Figure 3, control treatment), i.e. the least carotenoid amount was found at day 42 and the highest at day 132 after germination. Application of Artemisia extract significantly increased carotenoid amount in Bromus leaves. However, the only significant increase was at the early stage of growth, i.e. 42 days after Bromus seed germination. Extracts taken at different phenological stages of Artemisia had similar allelopathic effects on Bromus seedlings.

Oxidative stress indices (MDA, H$_2$O$_2$ and POD). Oxidative indices were affected by both Bromus phenological age and the Artemisia extracts (Figure 4). As Bromus was growing from early vegetative growth towards the flowering stage (day 42 - day 132, control treatment) MDA increased (Figure 4a), H$_2$O$_2$ did not vary (Figure 4b), but POD enzyme reduced (Figure 4c). Nevertheless, the oxidative factors showed similar responses to the allelopathic treatments. MDA, H$_2$O$_2$ and POD enzyme activity showed significantly higher values when treated by Artemisia extract, as compared with the control conditions. Furthermore, Artemisia extract taken at the early growth stage had greater allelopathic impacts than those taken at full vegetative and/or flowering stages (Figure 4).

DISCUSSION
This research was conducted in response to the contrary results that had been found between the field and glasshouse observations. Field observations indicated natural establishment of Bromus under the canopy of Artemisia (Jankju et al., 2008), whereas glasshouse experiments had indicated inhibitory effects of Artemisia extracts on seed germination, seedling growth and physiology of Bromus seedlings (Behdad et al., 2011).

Allelopathy and Phenology
Bromus seeds germinate in early autumn (October to mid-November), when the physiological data indicate lowest allelopathic effect by Artemisia. Mature individuals of Bromus start their early vegetative growth at late-March to early-April, when Artemisia is dormant. Moreover, about 40 percent of the annual rainfall in Baharkish rangelands occurs during March-May (Jankju et al., 2008), which can wash away the allelochemicals compounds from the Artemisia understory and provide a more favorable microclimate condition for Bromus. In a study in Baharkish rangelands, Jankju et al., (2008) found facilitative effect of Artemisia for establishment of transplanted Bromus seedlings under its canopy. In the same area, Maghamnia et al., (2009) found early season (April – May) facilitation for temperature and soil moisture but late season competition (June – July) for water, by Artemisia on Bromus. Accordingly, a one month gap in phenology of the two species, together with the early season facilitation by Artemisia, can provide an opportunity of natural establishment of Bromus under canopy of Artemisia.

The aging of Bromus intensified allelopathic stress of Artemisia extract on Bromus individuals, especially at the flowering stage. Chakrabarty et al., (2007) and Cabello et al., (2006) also found increase of reactive oxygen species (ROS) concentrations and lipid peroxidation by aging plant, which subsequently increased the oxidative stress enzymes and antioxidant compound activity. As plants get older, chlorophyll degradation becomes more rapid than carotenoids degradation and hence the amount of enzymes and antioxidant compounds are increased (Hai-Chun et al., 2002); so that ROS as an usual signal has an important role in controlling gene expression during plant senescence (Navabpour et al., 2003).

Allelopathic stress and Bromus responses
Shoot extracts of Artemisia reduced amount of chlorophyll a, b and total chlorophyll in Bromus leaves. The exact mechanism for the reduction of chlorophyll content in plants treated by allelochemical compounds is not clearly known, but it can be due to the inhibition of chlorophyll biosynthesis, the increase in chlorophyllase enzyme
activity or both of them (Shalinder and Batish, 2010). Some phenolic compounds, such as coumaric and ferulic acids induce chlorophyllase enzyme activity (Mighani, 2003). The reduction of leaf chlorophyll content can also be due to an increase in the metabolic processes that are related to the synthesis of new pigments (Al-Juboory and Ahmad, 1994; Babu and Kandasamy, 1997). Accordingly, the reduction of chlorophyll contents in Bromus seedlings can be due to effects of phenolic compounds which exist in aqueous extract of Artemisia shoots (see also Behdad et al., 2009).

Carotenoid content was increased in leaves of Bromus, when treated by Artemisia shoot extracts. Similar to our results, Grassmann (2005), El-Rokiek and Eid, (2009) and Omidpanah et al., (2009) found that the allelopathic compounds may increase antioxidants such as carotenoid pigments. Carotenoids are plant pigments that act as antioxidant and are essential compounds of photosynthetic apparatus. They are also involved in the elimination of ROS in photosynthetic complexes (Howltt and Pogson, 2006). An increase in carotenoid content under the allelopathic treatments, by increasing Bromus age, can be attributed to its antioxidant property and protective role for photosynthetic membrane under the stress conditions (Omidpanah et al., 2011).

There was an increase in the amount of MDA and H$_2$O$_2$ in leaves of Bromus, under allelopathic treatments. Allelochemicals can produce various kinds of ROS and oxidative stresses (Mutlu et al., 2010). In the cell membrane, oxidation of phenolic compounds produces large quantities of quinon that is toxic and responsible for producing reactive oxygen (Haddadchi and Gerivani, 2009). MDA is released as a result of cell membrane damages, therefore its increase in the tissue of Bromus seedlings may indicate cell membrane degradation due to the allelopathic stress. Adding allelopathic compounds to the hydroponic culture media degraded cell membrane and reduced seedlings growth of cucumber (Yu et al., 2003). Leaf extract of Hemistepta lyrata (an allelopathic species) increased the accumulation and MDA content in cucumber and radish (Xinxiang et al., 2009). In another study, Singh et al., (2006) found that exposing roots of Cassia occidentalis to alpha-pinene, induced peroxidation of cell membrane and oxidative stress, which subsequently increased the amount of H$_2$O$_2$ and MDA. Therefore, in this experiment, the increases in the amount of H$_2$O$_2$, membrane lipid peroxidation and MDA under the extract treatments can be related to the allelopathic stress of Artemisia on Bromus seedlings.

CONCLUSIONS

According to the results of this research, three possible explanations can be proposed for the coexistence of Artemisia khorassanica and Bromus kopetdaghensis in Baharkish rangelands. (1) A one month gap between the phenological stages of the two species may provide an opportunity for Bromus establishment under the canopy of Artemisia; Bromus starts its phenological stage when Artemisia is dormant and/or has lowest allelopathic effect. (2) Temporal shifts in allelopathic effects and responses: Bromus showed the highest physiological adaptation when Artemisia had the highest allelopathic effects (early vegetative). (3) Bromus can partially ameliorate the allelopathic stress. There was a declining trend in the amount of chlorophyll and H$_2$O$_2$ and an increasing trend in carotenoids, MDA content and POD enzyme activity as the allelopathic stress was increasing. Further, Bromus increased biomass investment on root under the allelopathic treatments. As a result, total dry weight of Bromus was not significantly different when treated by Artemisia extract in comparison with the control conditions.

<table>
<thead>
<tr>
<th>Dates</th>
<th>Artemisia</th>
<th>Bromus</th>
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<tbody>
<tr>
<td>15$^{th}$ March-20$^{th}$ April</td>
<td>Dormant</td>
<td>Early Vegetative</td>
</tr>
<tr>
<td>20$^{th}$ April – 30$^{th}$ June</td>
<td>Early Vegetative</td>
<td>Full Vegetative</td>
</tr>
<tr>
<td>July – early August</td>
<td>Full Vegetative</td>
<td>Flowering</td>
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<td></td>
<td></td>
<td>Dormant</td>
</tr>
<tr>
<td>Mid August – late September</td>
<td>Flowering and Seed shedding</td>
<td>Dormant</td>
</tr>
<tr>
<td>October – 15$^{th}$ November</td>
<td>(Seed germination for new seedlings)</td>
<td>(Seed germination for new seedlings)</td>
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<tr>
<td>15$^{th}$ November – 15$^{th}$ March</td>
<td>Dormant</td>
<td>Dormant</td>
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<tr>
<td></td>
<td></td>
<td>(Slow vegetative growth for new seedlings)</td>
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</tbody>
</table>
Figure 1. Effects of Artemisia extract on root shoot ratio of Bromus in different growth stages (Means indicated by similar letters are not significantly different at P<0.05).

Figure 2. Effects of Artemisia extract on concentration of Chlorophyll a in leaves of Bromus at different growth stages (Means indicated by similar letters are not significantly different at P<0.05).

Figure 3. Effects of Artemisia extract on concentration of Carotenoid in leaves of Bromus at different growth stages (Means indicated by similar letters are not significantly different at P<0.05).
Figure 4. Effects of Artemisia extract on Malondialdehyde (MDA) concentration (a), Hydrogen peroxide concentration (b) and Peroxidase enzyme activity (c) in leaves of Bromus at different growth stages. (Means indicated by similar letters are not significantly different at P<0.05).

REFERENCE


2008