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Short communication

A novel concept for the control of parasitic weeds by decomposing germination stimulants prior to action

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ABSTRACT

A new concept for controlling parasitic weeds is described. By decomposing germination stimulants prior to action no germination of seeds can take place anymore. Ethanol fractions of the strigolactone (SL) analogues viz., the standard synthetic analogues GR 24 and Nijmegen-1, and analogues derived from tetralone and coumarine, were added to an aqueous buffer with a pH ranging from 6 to 8 and the half lifes ($t_{1/2}$) of the hydrolysis were measured. Nijmegen-1 hydrolysed faster than GR 24 and the analogue from tetralone was the most stable one at all pH's. It was found that the aqueous solutions of either borax or thiourea rapidly decompose typical SL analogues, including GR 24 and Nijmegen-1, within an hour. The hydrolysis of SLs by borax was monitored with UV spectroscopy and for thiourea gas chromatography was used. This decomposition of SLs by either borax or thiourea in natural conditions would deprive the seeds of the parasitic weeds of the essential germination stimulants and as a consequence not allow them to germinate. Hence, conditions for an effective weed control are fulfilled.

1. Introduction

Parasitic weeds, especially *Striga* spp. and *Orobanche* spp., are increasingly becoming a threat to agricultural food production in many countries (Parker, 2009, 2012). These weeds attack a wide range of important food crops, thus directly affecting the food and nutrition security of humans and cattle in Africa, Asia and the Mediterranean region. Due to global warming induced changes in temperature and distribution of rainfall, these parasites have invaded new places like Southern Europe, thereby causing severe damage to important food crops (Grenz and Sauerborn, 2007). *Striga* and *Orobanche* are obligatory in nature, meaning that they entirely depend on the host plants for their nutrients and mineral needs. It is known for a long time that seeds of these parasites require stimulants present in host root exudates to germinate, ensuring that they germinate only in the presence of a host plant to which the germinated seed will attach itself in order to obtain essential ingredients for development. This is a naturally evolved mechanism for survival of these parasitic plants (Brown et al., 1949, 1951; Yoneyama et al., 2009; Xie et al., 2010). The first germination stimulant was isolated from root exudates of cotton (a non-host plant) by Cook et al. (1966). The structure of this stimulant, which was named strigol (**1**) (Fig. 1), was firmly secured twenty years later (Brooks et al., 1985). The isolation, identification and elucidation of natural germination stimulants, which collectively are named as strigolactones (SLs), is very difficult due to the fact that only minute amounts are exuded by plants, ca 15 pg per day per plant (Humphrey and Beale, 2006). At present, a whole series of natural SLs has been identified and characterized; typical examples are sorgolactone (**2**) (Fig. 1), orobanchol and solanacol (Yoneyama et al., 2009; Xie et al., 2010, 2013). The SLs invariably contain three annelated rings (the ABC scaffold) connected with a butenolide (D-ring) via an enol ether bridge (see structure of strigol (**1**)). Extensive structure–activity studies revealed that the bioactiphore of SLs resides in the CD part of the molecules (Mangnus and Zwanenburg, 1992; Zwanenburg et al., 2009; Zwanenburg and Pospíšil, 2013). This information allowed the design and synthesis of SL analogues with a much simpler structure than the natural SLs, but retaining the bioactivity (Nefkens et al., 1997; Zwanenburg et al., 2009; Zwanenburg and Pospíšil, 2013). The first series of SL analogues is the GR series described by Johnson et al. (1981). GR 24 (**3**) (Johnson et al., 1981; Malik et al., 2010) is most well-known and used as a standard in germination assays. Other successful examples are Nijmegen-1 (Nijm) (**4**) (Nefkens et al., 1997), a tetralone derived SL analogue Tet, (**5**) (Mwakaboko and Zwanenburg, 2011a) and a coumarine derived SL analogue Cou (**6**) (Mwakaboko and Zwanenburg, 2011b). (For the respective structures, see Fig. 1.)

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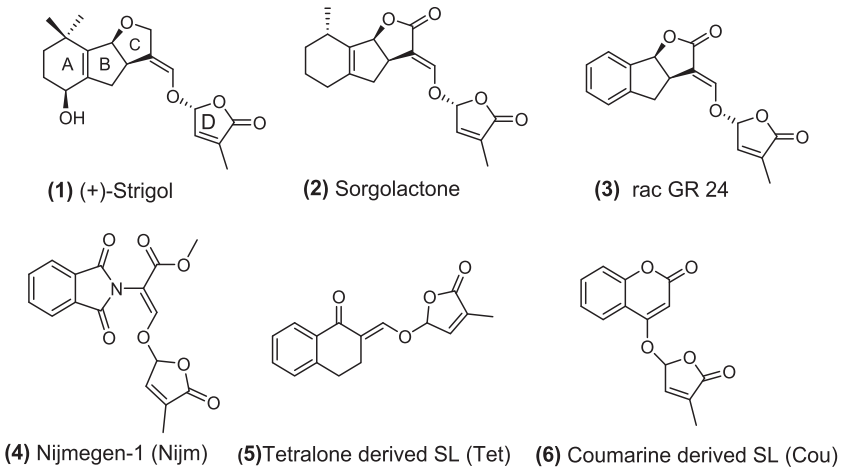

Fig. 1. Structures of some natural SLs and some synthetic SL analogues.

Several strategies for the control of these parasitic weeds have been considered; however none of them could provide fool-proof protection against their invasion and damage (Joel, 2000; Rubiales et al., 2009; Parker, 2009, 2012; Hearne, 2009; Rodenburg et al., 2010). A problematic aspect is that these weeds produce very large number of seeds that can remain viable in the soil for up to twenty years (Rubiales et al., 2009). An old control method of parasitic weeds is hand weeding, which is both labour intensive and ineffective. Crop rotation, the use of false hosts (trap crops) and catch crops can be effective to some extent, but require a strict agricultural regime (Hooper et al., 2009). Breeding of resistant crops requires exhaustive selection techniques; however unfortunately, the resistance disappears after a period of *ca.* five years (Pacureanu-Joita et al., 2009). Chemical control by herbicides can be effective in controlling the weeds, however, host plants may be affected as well and the method has been criticized for its environmental effects, especially when non-selective and highly toxic chemicals are used (Parker, 2009, 2012; Rubiales et al., 2009; Hearne, 2009).

It had long been realized (Johnson et al., 1976) that if a germination stimulant would be applied to the soil before the host crop is planted; the parasitic seeds would germinate in the absence of a host, but would not survive as the necessary attachment to the host plant is not possible. This method is commonly referred to as the "suicidal germination approach". The early successful attempts with GR 7 (this is GR 24 lacking the aromatic A-ring) to achieve suicidal germination were stopped due to lack of financial support and availability of sufficient amounts of stimulant. However, recently, SL analogues have successfully been used in pot experiments to induce suicidal germination (Kgosi et al., 2012). A successful suicidal germination in the field was reported using Nijmegen-1 as SL analogue in tobacco infested by *Orobanche cumana* (Zwanenburg et al., 2009). In these experiments the stimulant was formulated in an emulsion which prevents untimely hydrolysis of the stimulant and leaching down to lower soil layers.

It has been suggested by several authors that the limited stability of stimulants in soil would preclude their use in the suicidal germination approach (Babiker and Hamdoun, 1982). It was observed that the stability of GR compounds GR 24 and GR 7 was dependent of several soil factors mainly pH, with decreased stability in basic soils (Johnson et al., 1976; Babiker et al., 1988) and also from excess of moisture leading to decreased response of seeds to these SL analogues (Babiker et al., 1987).

The aim of this paper is to present a new concept for the control of parasitic weeds making use of the instability of germination stimulants under certain conditions.

2. Materials and methods

The germination stimulants viz., GR 24 (**3**), Nijm (**4**), Tet (**5**) and Cou (**6**) were prepared as described previously (Malik et al., 2010; Nefkens et al., 1997; Mwakaboko and Zwanenburg, 2011a, 2011b, respectively). Borax (Sodium tetraborate decahydrate) and thiourea were purchased from Sigma. Thin layer chromatography (TLC) was performed using Merck Silicagel 60F254 TLC plates and ethyl acetate/heptane 2:1 as eluent. UV spectra were recorded using a Jasco V630 spectrophotometer and for Gas–Liquid Chromatography (GLC) a Shimadzu 2010 Plus instrument was used. For the stability experiments, buffers were prepared from 5 mM ammonium acetate and adjusting the pH from 6 to 8 at 0.5 intervals by adding 0.5 M NaOH. The SL analogues were dissolved in ethanol (1 mM) and 50 μL were placed in a 20 ml vial, ethanol was removed in vacuo and buffer was added. Aliquots were taken at regular intervals (4 h, 1 d, 2 d, 1 w, 2 w) and analysed by Liquid Chromatography/Mass Spectrometry (LCMS). The $t_{1/2}$ values (Table 1) were determined from a plot of concentration vs time.

3. Results and discussion

We analysed the hydrolytic stability of some SL analogues at various pH values. It was found that at alkaline pH the stability of these analogues rapidly decreases. The results are collated in Table 1.

The results indicate that Tet (**5**) has the highest $t_{1/2}$ at all pH and is the most stable of the four SL analogues tested: 20 h even at pH 8.0. Cou (**6**) has a $t_{1/2}$ in the middle range and hydrolysis is of

Table 1
Stability of some SL analogues at various pH values.

Compounds/pH	Half-life ($t_{1/2}$ in h)				
	6.0	6.5	7.0	7.5	8.0
GR 24 (**3**)	140	120	100	42	5
Nijm (**4**)	35	22	10	<4	<4
Tet (**5**)	–	210	170	95	20
Cou (**6**)	90	75	45	13	<4

Table 2
Time (in h) required for complete disappearance of SL stimulants (concentration 5 mM) when treated with aqueous alkaline solutions or thiourea.

Stimulants	Sodium bicarbonate (mM)				Sodium acetate (mM)				Sodium thiosulfate (mM)				Borax (mM)				Thiourea (mM)			
	5	25	50	100	5	25	50	100	5	25	50	100	5	25	50	100	5	25	50	100
Nijm (**4**)	>24	>24	14	8.0	>24	>24	7.5	3.0	>24	>24	12	6.0	>24	>24	4.0	0.5	>24	>24	4.0	2.0
GR24 (**3**)	>24	>24	12	5.0	>24	>24	6.0	2.5	>24	>24	14	8.0	>24	>24	5.5	1.0	>24	>24	6.0	2.5

not much significance in bioassays. In the pH ranging from 7.5 to 8.0 rapid loss of hydrolytic stability is observed for all the four stimulants studied. It is noteworthy that Nijm (**4**) is hydrolysed faster than GR 24 (**3**) but, it was shown in field experiments with Nijm (**4**) that appropriate formulation prevents untimely hydrolysis and that the suicidal approach is still feasible (Zwanenburg et al., 2009). The stability of SLs towards hydrolysis was reported twice in the recent literature. Akiyama et al. (2010) measured the half-life in water containing 3% of methanol ($t_{1/2}$ = 10 d), the pH was not specified and Boyer et al. (2012) in ethanol:water 1:4 at pH 6.7 ($t_{1/2}$ = 14 d). As shown in Table 1 the pH has a great influence. Methanol and ethanol slow down the hydrolysis considerably (Boyer et al., 2012).

Looking at the stability at varying pH values it occurred to us that this disadvantage of increasing instability towards hydrolysis at higher pH can be used to our advantage. When the stimulants exuded by the roots of the host plant are decomposed as soon as they leave the root by exposure to an alkaline pH, no effective germination is possible any more. In order to materialize this idea, we studied the decomposition of stimulants using aqueous alkaline solution, viz., sodium bicarbonate, sodium acetate, sodium thiosulfate, thiourea and borax by monitoring the hydrolysis with TLC. The most appropriate basic solution turned out to be borax ($Na_2B_4O_7$), which has a pH of 9.2–9.3 at varying concentrations. The ratio of stimulant and borax concentration was varied and the fastest clearance of the stimulant was observed using the ratio of stimulant and borax of 1:100. The times needed for complete disappearance of the stimulants at various concentrations of different agents are given in Table 2. These TLC monitoring experiments gave a good first indication of the time to complete the hydrolysis of the stimulant, however we were limited in the concentration as a minimum of 5 mM is needed to visualize the compounds on the TLC plates.

In order to investigate the decomposition at stimulant concentrations approaching that of the naturally occurring SLs in the root exudates, we monitored the rate of hydrolysis by borax using UV spectroscopy. The concentration of the SL analogues amounted to 10^{-7} M, near to the concentration in the natural conditions and that of borax to 10^{-5} M. At a wavelength of 220 nm the difference in absorption between beginning and end was sufficient to monitor the hydrolysis, except for Tet (**5**). The curves of absorption *vs* time are shown in Fig. 2.

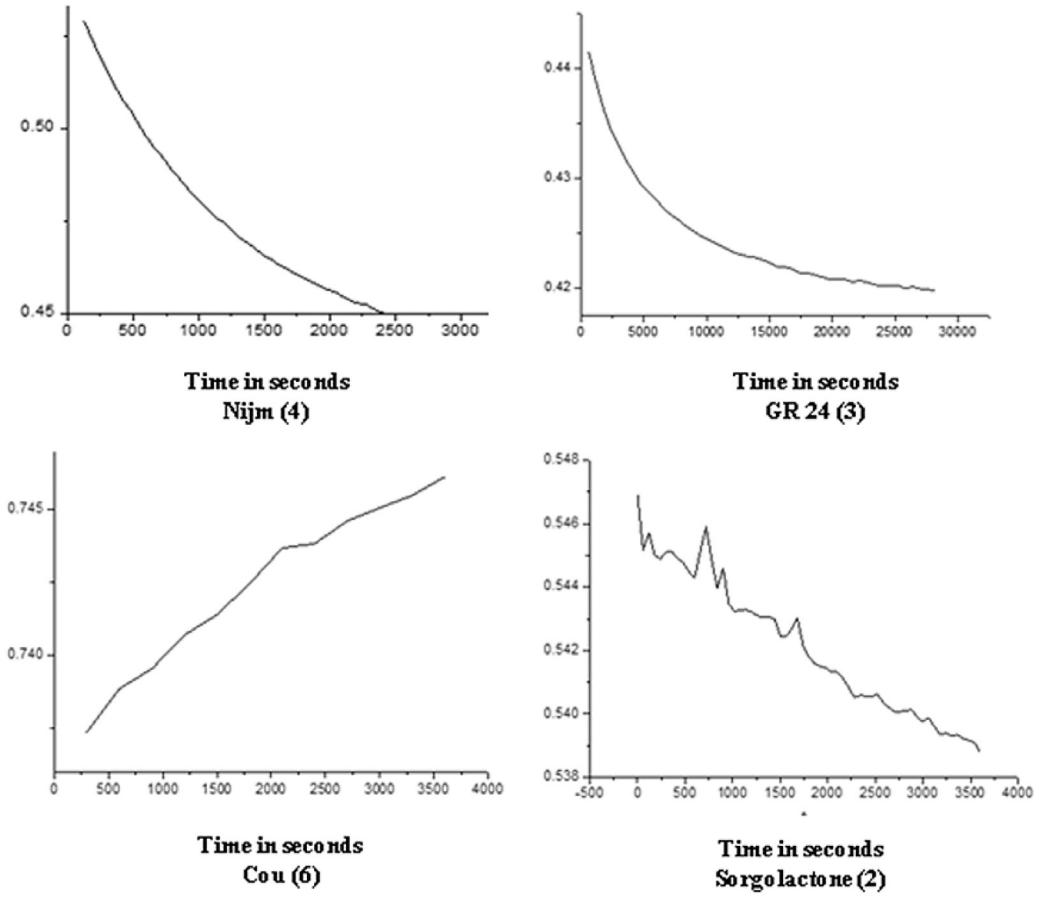

Fig. 2. Kinetics of the decomposition of some SL analogues and sorgolactone in presence of borax (SL concentration 10^{-7} M, borax 10^{-5} M).

Nijm (**4**), GR 24 (**3**) and sorgolactone (**2**) had higher absorbance at the initial state and subsequently losing their absorbance after hydrolysis with borax. In case of Cou (**6**), the initial state had a lower absorbance than the final hydrolysed product after reacting 3500 s with borax. For Nijm (**4**) the clearance time, i.e. the time needed to fully decompose the stimulant, was 2400 s, for GR 24 (**3**) 2500 s, and for Cou (**6**) 2800 s. Also the natural stimulant sorgolactone (**2**) (Sugimoto et al., 1998) was included in this study (clearance time 2500 s). For Tet (**5**) the clearance time of 2 h was estimated from the TLC experiments.

The clearance time of different stimulants by borax in a concentration ratio of 1:100 gives a clear picture of the instability towards hydrolysis of the compounds in the presence of borax. It is relevant to mention that the pH of borax solutions does not differ very much with concentration, making this agent attractive for field applications to control parasitic weeds. In addition, borax is an inexpensive and eco-friendly salt (Castro and Brighenti, 2007), which adds to the attractiveness of this controlling agent. Thus, the current study demonstrates that borax can be used to decompose germination stimulants prior to their interaction with seeds of parasitic weeds, being conceptually a method for controlling of the parasitic weeds, such as *Orobanche* spp. For field applications formulation of borax is necessary and has to be developed keeping in mind the differences in properties of various types of soil in which these parasitic weeds are observed. If this is done properly the formulated salt can also form a film of borax emulsion around the seeds. This would be of help in case some stimulant would reach the seeds in case it had escaped the hydrolysis at the site of the root, it will be hydrolysed before entering the seeds. In a practical sense this is an example of *double gate keeping*: decomposition of stimulant when exuded from the roots and when approaching the seeds.

In spite of the fact that borax has attractive features in decomposing SLs prior to action, on the long run its continuous use may give rise to too high boron concentrations in the soil, resulting in undesirable soil intoxication. Therefore, we also considered an alternative agent for rapid decomposition of SLs. For this we selected a renowned nucleophilic agent, namely thiourea [$(H_2N)_2C{=}S$]. This compound will react in its iminothiol tautomeric form with the α,β-unsaturated enone unit as regular thiols do (Mangnus and Zwanenburg, 1992), leading to detachment of the D-ring and accordingly to deactivation of the SL stimulant. As shown in Table 2, thiourea indeed reacts rapidly with the stimulants and can give complete disappearance of the SL provided it is used in excess, at least 20×. The reaction with thiourea cannot be monitored with UV due the absorption of excess thiourea at the same wavelength range (200–230 nm) of the stimulants. Thus, measuring the clearance time of stimulant at a concentration in the range of that natural SLs when exuded by host plants (10^{-7}–10^{-9} M), requires a different technique. For this Gas Liquid Chromatography (GLC) was found appropriate. The SLs are stable during the GLC analysis. The reaction is in fact too fast to follow the kinetics. The clearance time ranging from 30 to 60 min was determined for the SLs using a 50× excess of thiourea. Gratifyingly, the results demonstrate that thiourea fulfils the requirement of a short clearance time and therefore it is a potential controlling agent, in the same way as described for borax. Thiourea can easily be formulated, it is an inexpensive eco-friendly compound as well and a bio-regulatory molecule for plant growth stimulation (Mathur et al., 2006), and acts as an antioxidant in plant protection (Pandey et al., 2013).

In conclusion, we developed a novel concept for the control of parasitic weeds by decomposing the germination stimulant prior to action. For this, we identified two attractive controlling agents, namely aqueous solutions of borax and thiourea.

Currently, *in vivo* studies including field applications are under investigation in India (Directorate of Weed Science Research, Jabalpur, M.P. India) to elaborate these concepts in actual practice.

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