Effects of carbon dioxide on the searching behaviour of the root-feeding clover weevil *Sitona lepidus* (Coleoptera: Curculionidae)

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Abstract

The respiratory emission of CO_2 from roots is frequently proposed as an attractant that allows soil-dwelling insects to locate host plant roots, but this role has recently become less certain. CO_2 is emitted from many sources other than roots, so does not necessarily indicate the presence of host plants, and because of the high density of roots in the upper soil layers, spatial gradients may not always be perceptible by soil-dwelling insects. The role of CO_2 in host location was investigated using the clover root weevil *Sitona lepidus* Gyllenhall and its host plant white clover (*Trifolium repens* L.) as a model system. Rhizochamber experiments showed that CO_2 concentrations were approximately 1000 ppm around the roots of white clover, but significantly decreased with increasing distance from roots. In behavioural experiments, no evidence was found for any attraction by *S. lepidus* larvae to point emissions of CO_2, regardless of emission rates. Fewer than 15% of larvae were attracted to point emissions of CO_2, compared with a control response of 17%. However, fractal analysis of movement paths in constant CO_2 concentrations demonstrated that searching by *S. lepidus* larvae significantly intensified when they experienced CO_2 concentrations similar to those found around the roots of white clover (i.e. 1000 ppm). It is suggested that respiratory emissions of CO_2 may act as a 'search trigger' for *S. lepidus*, whereby it induces larvae to search a smaller area more intensively, in order to detect location cues that are more specific to their host plant.

Keywords: fractal analysis, orientation, semiochemicals

Introduction

The role of soil-dwelling insect herbivores in ecosystem processes is becoming of interest to a wide range of applied and ecological researchers (Van der Putten *et al.*, 2001; Wardle *et al.*, 2004; Bardgett, 2005; Rasmann *et al.*, 2005).

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Soil-dwelling insects can account for reductions in crop yields of 40–50% (Spike & Tollefson, 1991), and they have been shown to drive plant (Schadler *et al.*, 2004), microbial (Treonis *et al.*, 2005) and above-ground insect herbivore community dynamics (Blossey & Hunt-Joshi, 2003). Despite their significance, important fundamental aspects of their biology remain poorly understood compared with insect herbivores living above-ground. In particular, the chemical ecology underpinning host plant location by soil-dwelling insects has received limited attention, hindered in part by the difficulty associated with visualizing these interactions in soil.

The most commonly cited mechanism underpinning root location by soil-dwelling insects is the respiratory emission of carbon dioxide from roots. Waste products such as CO_2 are ideal candidates for such signalling mechanisms, as plants are unable to switch off such metabolic functions while remaining active. Over 20 studies to date have reported that soil-dwelling insects are attracted to CO_2 (Johnson & Gregory, 2006), and this has led to the conclusion that CO_2 is a major means of root location by such insect herbivores (e.g. Mortimer *et al.*, 1999). However, there are a number of arguments for questioning the exact role of CO_2 in such processes (Johnson & Gregory, 2006). Firstly, emissions of CO_2 occur from numerous sources in the soil and are ubiquitous, so this mechanism does not permit insects to discriminate between plant species, or even between root and microbial emissions of CO_2. Secondly, the extremely high density of roots in the upper layers ($>1\,cm\,cm^{-3}$; Gregory, 1988) means that the sources of CO_2 are in extremely close proximity (typically $<10\,mm$ apart) which could make spatial distinction between sources difficult. Finally, because soil contains much more CO_2 than the atmosphere, vertical gradients of CO_2 will always be stronger than horizontal gradients from root surfaces (Gollany *et al.*, 1993; Sheppard & Lloyd, 2002) which could also confound spatial perception of root emissions by insects. So while it is questionable whether CO_2 acts as a host plant attractant for all root feeders, it may still play a role in enabling these herbivores to locate roots. For instance, other soil-dwelling organisms such as nematodes react to location cues released by a host by either moving directly towards the source along the gradient (chemotaxis), or else changing locomotion (e.g. turning patterns) in response to the stimulus without orientating towards the source (kinesis) (Dusenbery, 1992).

The clover root weevil, *Sitona lepidus* Gyllenhall (Coleoptera: Curculionidae), which feeds specifically on white clover (*Trifolium repens* L.) was investigated in this study. As an adult, *S. lepidus* feeds above-ground on leaves, where the maternal insects lay many thousands of eggs that give rise to soil-dwelling larvae that then attack the root system. Neonatal larvae are small (~1 mm long) and must locate host plant roots to avoid starvation. The aims of this study were: (i) to quantify the CO_2 conditions around white clover roots; (ii) to investigate whether neonatal *S. lepidus* larvae were attracted to point emissions of CO_2; and (iii) determine whether different CO_2 concentrations affected the searching behaviour of neonatal larvae.

For the third aim of this study, we used fractal analysis to quantify movement behaviour. Within strict upper and lower scale boundaries, fractal analysis has been found to be a rugged quantifier of organism movement, providing an indication of searching patterns of organisms against a range of environmental perturbations (Doerr & Doerr, 2004; Halley *et al.*, 2004; Nams, 2005). There are a number of methods for defining fractal dimension (Halley *et al.*, 2004). In this paper we adopt the method used by Doerr & Doerr (2004) and Nams (2005), which is based on the seminal method proposed by Mandelbrot (1967) to measure the length of a trajectory using a polygonal path with side length ε. The fractal dimension, *D*, of the trajectory (or movement path) is defined as:

$$L(\varepsilon) = k\varepsilon^{1-D} \quad D \ge 1, \tag{1}$$

where $L(\varepsilon)$ is the total trajectory length and k is a positive constant. When $D = 1$ the animal is following a linear trajectory, as D values increase this reflects increasingly tortuous trajectories, conventionally reaching a maximum of $D = 2$, but higher values are possible. In the context of animal searching behaviour in a two-dimensional arena used in the current study, the range of D is between 1 and 2. Values of D closer to 1 reflect animal movements between resource patches, whereas D values closer to 2 reflect more intense searches of smaller areas within a potential resource patch (Doerr & Doerr, 2004).

Materials and methods

CO₂ concentrations in the root zone

To measure CO_2 concentrations at different distances from roots, five rhizochambers comprising plant and soil compartments were used (fig. 1 inset). The mesh interface separating the two compartments prevented roots growing into the soil compartment but permitted transmission of gaseous and aqueous compounds. The soil compartment of the rhizochambers were filled with 1.12 kg sterilized and sieved soil (particles < 2 mm; dry soil bulk density 1.24 $Mg\,m^{-3}$). White clover (cv. Gwenda) plants were grown from seed in John Innes no. 2 compost, before being transferred to the plant compartments of the rhizochambers and soil (details as above) packed around them. Plants were clones of one another and of a similar size and physiological condition. Alkathene beads were placed on the soil surface to minimize evaporation and sides of the rhizochambers were covered with metallic foil to prevent algal growth. Rhizochambers were maintained in a glasshouse (20 ± 5°C), where they were kept on balances and watered evenly to have a gravimetric water content of 20%. Each week all plants were provided with 25 ml of a modified Arnon's solution (Hewitt, 1966; Hatch & Murray, 1994). After 6 weeks, the concentrations of CO_2 were measured (see fig. 1 inset) in the rhizosphere and at 2, 4 and 6 cm from the plant roots. These relate to distances from roots that *S. lepidus* larvae can 'perceive' host plant roots (Johnson *et al.*, 2004a). Measurements of CO_2 concentrations were made using a membrane-inlet mass-spectrometer (Hiden HPR-40, Hiden Analytical, Warrington, UK). A 1 mm diameter stainless steel probe with a perforated end covered in a silicon sheath, was inserted to a depth of 50 mm at each sampling location (fig. 1 inset) and CO_2 concentration measured five times at *m/z* 44.

Tracking experiments

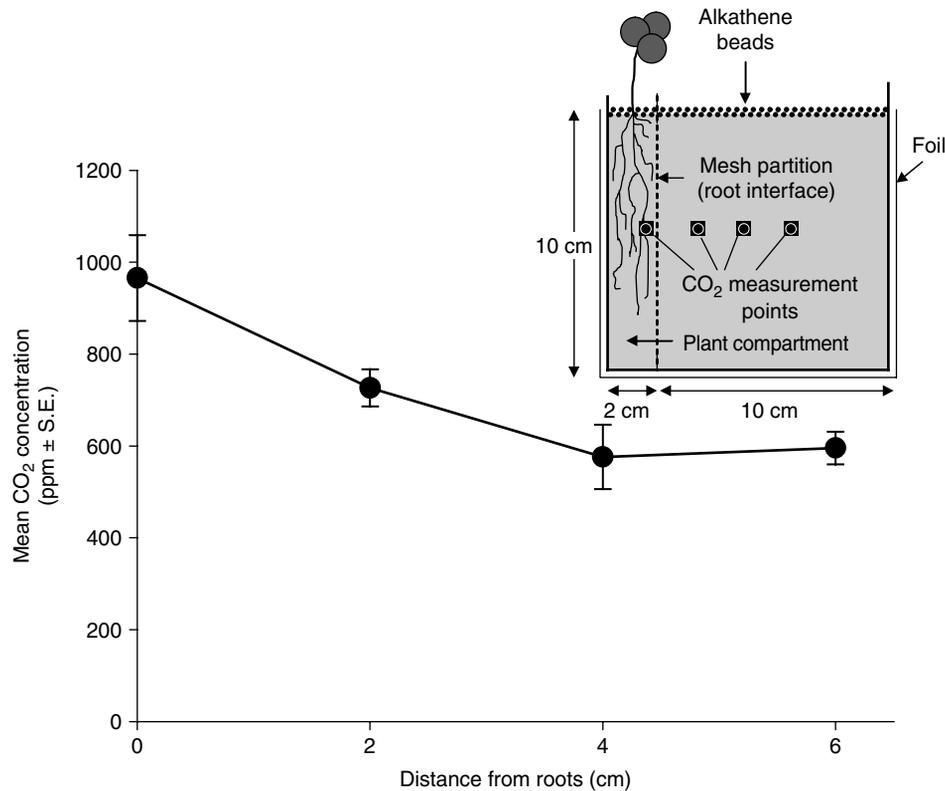Bioassays were conducted in circular arenas (diameter 90 mm) lined with moistened (1.5 ml distilled H_2O) filter

Fig. 1. Mean CO_2 concentrations with increasing distance from the root interface for white clover plants growing in rhizochambers (see inset), showing a statistically significant decline in CO_2 concentration ($r^2 = 0.49$, $P > 0.001$).

papers (fig. 2 inset) inside an insulated darkened chamber (light intensity: $< 0.05\,\mu mol\,m^{-2}\,s^{-1}$, temperature: $20 \pm 1°C$). A single neonatal larva was placed in the centre of the arena and the arena sealed with a Perspex lid. Larval movement was tracked using a digital camera (Pro-150ES, Pixera Corp., California, USA) above the arena. Images (exposure 600 ms) were relayed at 10 s intervals for 15 min to an image analysis unit incorporating a tracking algorithm (Image-Pro Plus^{TM} v. 5, Media Cybernetics Inc., Maryland, USA). Thirty replicates were conducted using fresh filter paper and larvae on each occasion. Two experiments were carried out to investigate: (i) whether *S. lepidus* larvae were attracted to point emissions of CO_2 at different flow rates, and (ii) whether *S. lepidus* movement patterns changed in constant CO_2 atmospheres.

The first experiment introduced CO_2 through an aperture at the side of the arena at four flow rates (5, 8, 16 and $32\,ml\,h^{-1}$) with the same volume of air drawn out from an aperture at the opposite end of the arena (fig. 2a). These flow rates and the experimental system itself were similar to those used by Jones & Coaker (1977) to measure the attraction of the carrot root fly, *Psila rosae* (Fabricius) (Diptera: Psilidae), to CO_2. The control consisted of CO_2-free air ($8\,ml\,h^{-1}$) being pumped into the arena and the same volume drawn out from the opposing aperture.

The second experiment altered the concentration of CO_2 in closed arenas by filling the arenas with different concentrations of CO_2 and air. CO_2 concentrations consisted of: 380 ppm, 1000 ppm and 2500 ppm ($\pm 5\%$) (BOC, Guildford, UK). A control was also conducted by flushing the arena
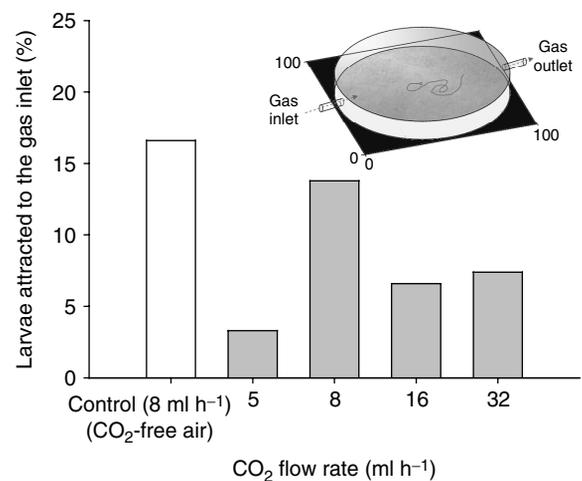

Fig. 2. Movement of *Sitona lepidus* larvae to within 10 mm of the gas inlet aperture in arenas, introducing either CO_2-free air (the control) or CO_2 at different flow rates. There were no statistically significant differences in the numbers of individual larvae attracted to the control (N = 30) and to emissions of CO_2 for flow rates of $5\,ml\,h^{-1}$ (N = 30, $P = 0.19$), $8\,ml\,h^{-1}$ (N = 29, $P = 0.99$), $16\,ml\,h^{-1}$ (N = 30, $P = 0.42$) or $32\,ml\,h^{-1}$ (N = 27, $P = 0.42$).

with CO_2-free air. Once the volume of the arena was replaced with the desired gas, a single neonatal larva was placed in the middle of the dish via an aperture at the side of

the arena (fig. 2a). Both apertures were immediately sealed and the visual tracking initiated.

Statistical analysis

Standard regression was used to analyse the relationship between CO_2 concentration and distance from roots. Fisher's exact P test was used to analyse attraction of larvae to point emissions of CO_2. Comparisons between larval speeds in the control and CO_2 treatments were analysed using t-tests. The power-law relationship for fractal analysis was tested using regression analysis. Fractal dimension values (D) were analysed with ANOVA tests, using an unbounded Johnson transformation (Chou *et al.*, 1998) because these data did not conform to normality. Tukey's tests were employed for *post hoc* comparisons. Approximately 3% of larvae tested were behaviourally inactive (perhaps through being damaged during manipulation) and were therefore not included in the analysis.

Results

CO_2 concentrations in the rhizosphere and tracking experiments

Carbon dioxide concentration in the vicinity of white clover roots was around 1000 ppm and showed a statistically significant decline in CO_2 concentrations with increasing distance from roots to around 600 ppm (fig. 1).

Experiments with a point emissions of CO_2 entering the arena showed that there was no statistically significant difference in the number of individual larvae moving within 10 mm of the gas inlet aperture when either CO_2 or CO_2-free air was pumped into the arena (fig. 2). The flow rate at which CO_2 entered the arena (5, 8, 16 or 32 ml h^{-1}) had no significant effect on larval behaviour. Larval movement speeds were slower in the presence of CO_2 compared with the control (mean speed = 0.22 mm s^{-1}) and significantly so for CO_2 flow rates of 5 ml h^{-1} ($t_{38} = 4.16$, $P < 0.001$) and 16 ml h^{-1} ($t_{57} = 2.06$, $P = 0.044$), where they displayed mean speeds of 0.16 and 0.18 mm s^{-1}, respectively.

The movement behaviour of *S. lepidus* larvae in the second experiment involving constant CO_2 concentrations was quantified using the fractal method described in detail by Nams (2005). Unlike the studies described by Nams (2005) and Doerr & Doerr (2004), our experimental organisms did not encounter habitat heterogeneity so it was predicted that the fractal dimension would be scale invariant. This was indeed the case, and fig. 3 demonstrates a power-law relationship between the number of dividers and divider size.

The mean fractal dimension values (D) for insects moving within CO_2 concentrations show that there was a statistically significant trend for higher values in arenas with CO_2 concentration of 1000 ppm than for arenas with 380 and 2500 ppm (fig. 4). The difference between 1000 and 0 ppm was also apparent ($P = 0.086$). This indicates that neonatal larvae are making more tortuous and intensive searching movements within a smaller area of the arena when moving in CO_2 conditions similar to those that would be experienced in the immediate vicinity of white clover roots (i.e. 1000 ppm) (fig. 1). Examples of *S. lepidus* larval movements at CO_2 concentration of 0 ppm (fig. 5a), 380 ppm (fig. 5b), 1000 ppm (fig. 5c) and 2500 ppm (fig. 5d) over 15 min illustrate this difference in searching intensity.
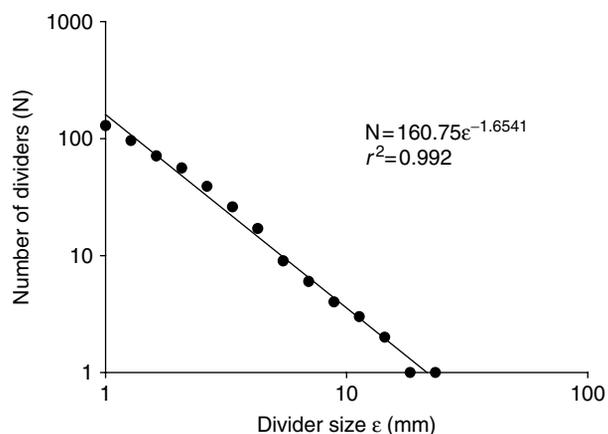

Fig. 3. The power-law relationship between trajectory length (number of dividers) and the divider length indicating that the fractal dimension for *Sitona lepidus* larvae is scale invariant in such arenas.
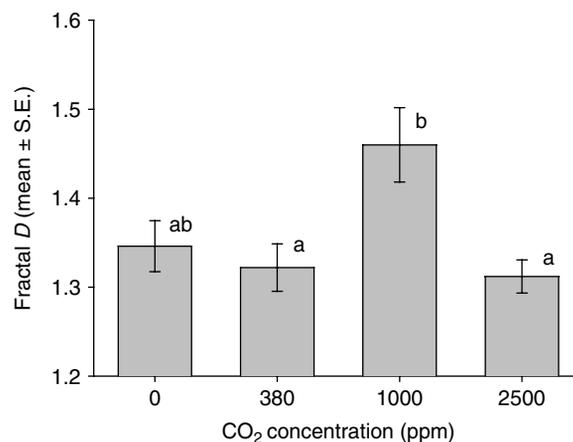

Fig. 4. Mean fractal dimension (D) values $\pm$ S.E. for neonatal *Sitona lepidus* larvae moving in closed arenas containing different CO_2 conditions, showing statistically significantly higher values for larvae in the 1000 ppm arena ($F_{3,102} = 4.08$, $P = 0.009$). Lower-case superscripts indicate significant differences between CO_2 concentrations ($P < 0.05$). The difference between 1000 ppm and 0 ppm approached statistical significance ($t = 2.39$, $P = 0.086$). 0 ppm: N = 27; 380 ppm: N = 25; 1000 ppm: N = 25; 2500 ppm: N = 29.

Discussion

The present study aimed to investigate the CO_2 concentrations around white clover roots, and how CO_2 affected the attraction and searching behaviour of a subterranean insect herbivore. Carbon dioxide concentrations in the immediate vicinity of white clover roots were *c.* 1000 ppm, which gradually decreased to *c.* 600 ppm >4 cm away. No evidence for the direct attraction of *S. lepidus* to CO_2 emissions was obtained, even though we used similar apparatus to that used by other investigators with other insects and replicated gradient conditions similar to those experienced by *S. lepidus* in natural conditions. However, the searching behaviour of neonatal larvae significantly intensified when larvae
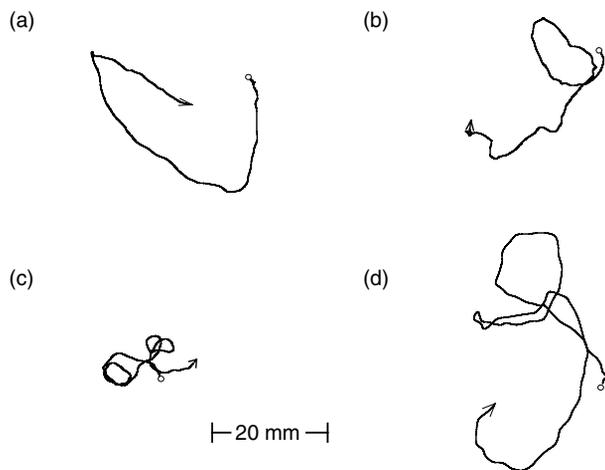
(a)   (b)

(c)   (d)

├─ 20 mm ─┤

Fig. 5. Examples of trajectories (and fractal D values) of neonatal *Sitona lepidus* larvae moving in CO$_2$ atmospheres of (a) 0 ppm, $D = 1.247$ (b) 380 ppm, $D = 1.289$ (c) 1000 ppm, $D = 1.677$ and (d) 2500 ppm, $D = 1.299$. Larval starting point indicated by a circle and direction of movement indicated by an arrow. The more tortuous path of *S. lepidus* larvae moving in CO$_2$ concentrations of 1000 ppm (c) is reflected in the high D value.

experienced CO$_2$ conditions that were typical of concentrations around white clover roots. In effect, searching intensified at 1000 ppm so that larvae conducted more 'thorough' searches of smaller areas compared with larvae that experienced lower or higher CO$_2$ concentrations. The fact that intensive searches were not seen at 2500 ppm could be due to inhibitory effects of high concentrations of CO$_2$, as previously reported for other root-feeding insects (Bernklau & Bjostad, 1998a).

The lack of evidence for direct attraction to CO$_2$ raises questions about whether respiratory emissions of CO$_2$ can be considered to be a general mechanism for root location by soil insect herbivores as previously suggested (Brown & Gange, 1990). Most reports of this simple attraction are concerned with polyphagous insects that feed on monoculture crops, for instance the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), feeding on maize (Bernklau & Bjostad, 1998b; Bernklau *et al.*, 2004). Carbon dioxide might be a more credible cue for such insects because CO$_2$ gradients may be stronger between bare strips of earth and strips of plants. Unlike the western corn rootworm, *Sitona lepidus* is *Trifolium* spp. specific and in particular shows a preference for white clover (*T. repens*) over companion plant species in mixed swards such as ryegrass (Johnson *et al.*, 2004a). Root exudates that are more specific to the host plant are likely to play a more important role for host location in such circumstances (Johnson *et al.*, 2005).

Although not an attractant, this study provides evidence that CO$_2$ may trigger more intensive and 'thorough' searches of smaller areas, perhaps in order to search for more specific chemical cues exuded by host plant roots. This could be analogous to the behaviour of the Australian tree creeper birds, in which lower fractal dimension values were found when they moved between resource patches but the fractal dimension rose sharply as they conducted more thorough searches within a resource patch (Doerr & Doerr, 2004). Respiratory emissions of CO$_2$ from roots could therefore indicate the potential proximity of roots and initiate more intensive searches of that area by *S. lepidus* larvae. The role of CO$_2$ as a general 'search trigger' is at least more congruent with the fact that CO$_2$ gradients in the soil would be of limited use for direct spatial orientation towards roots (see introduction). The generally slower larval speeds in the presence of CO$_2$ reported for the experiment with point emissions could be indicative of orthokinesis, a type of kinesis whereby the insect's speed alters in response to a stimulus (Dusenbery, 1992). It is unlikely that changes in speed were due to CO$_2$ induced anaesthesia because air was continually drawn through the arena thereby preventing a build-up of CO$_2$.

As with other studies with soil-dwelling organisms, the current behavioural experiments were by necessity conducted in soil-free apparatus in order to quantify subtle movements of larvae. Clearly, arenas have limitations in that they only allow movement in the lateral plane, whereas soil-dwelling insects can normally move in the vertical plane and their movement patterns will also be influenced by soil structure. However, there is currently no way of measuring insect behaviour in sufficient detail when they are moving within the soil matrix. X-ray tomography has been used to study coarse movements in the soil non-invasively (Harrison *et al.*, 1993; Johnson *et al.*, 2004b), but the interval between observations (a minimum of 30 min) does not allow subtle behavioural patterns to be quantified. So while arena experiments do not provide absolute data on insect burrowing patterns, they remain the most practical way of obtaining highly detailed comparative and unimpeded movement data.

Understanding the exact role of CO$_2$ in host plant location by soil-dwelling insects could have applied outcomes, since many of these insects are destructive agricultural and forestry pests (e.g. the black vine weevil). Moreover, the initial location of roots by newly born insects is normally the most vulnerable part of the life-cycle, so may provide the most opportune period for applying control measures. A recent trial with the western corn rootworm suggested that CO$_2$ could be used to disorientate or at least reduce the likelihood of locating maize roots (Bernklau *et al.*, 2004), so this could be feasible. In any case, these results suggest that the behavioural responses of subterranean insects to CO$_2$ emissions in the soil could be more complex than the simple attraction mechanisms hitherto proposed.

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