Reduction in nitrogen fixation by legumes following ALS herbicide application

**PROJECT DETAILS**

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<td>REDUCTION IN NITROGEN FIXATION BY LEGUMES FOLLOWING ALS HERBICIDE APPLICATION</td>
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**Summary**

Reduced nitrogen (N) fixation due to application of Group B herbicides is a potential problem. Half of the legume/herbicide combinations examined showed reductions in growth, nodulation and/or N fixation. Plant susceptibility was identified as a major mechanism reducing N fixation. Root morphology and proteomics work confirmed that herbicide effects on the plant are likely to have major consequences for symbiotic nitrogen fixation. Downshifting of nitrogen assimilation and deformation of root hairs and meristems are likely to disrupt rhizobial infection and nodule development/function. Herbicide tolerant legumes (e.g. Angel^ victims) may ameliorate reductions in N fixation due to herbicide application.

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Conclusions

Group B herbicides reduce nitrogen fixation in a range of legumes. Previous studies on chickpeas were extended to a number of grain and pasture legumes, and negative impacts on growth, nodulation and/or nitrogen fixation occurred in the majority of cases.

Extension of screening results would require further assessment in the field, since a myriad of climatic and edaphic factors will influence the outcome. We support the findings of project CSO00013 in this regard.

Susceptibility of the plant appears to be the major mechanism by which Group B herbicides inhibit nitrogen fixation. Although herbicide does not kill rhizobia in liquid culture, there appears to be a minor impact on the symbiosis via the rhizobia. The implications of this in a field situation are not yet fully understood.

Group B herbicides have the capacity to alter root morphology, particularly meristematic development and root hairs. This is likely to impede infection by rhizobia and development of nodules.

Group B herbicides elicit a plant response which involves stress response proteins and changes to primary metabolism. Specifically, a major protein involving nitrogen assimilation was down-regulated, with likely implications for nitrogen fixation.

A herbicide tolerant medic variety, FEH-1 or ‘Angel’\(^1\) had increased biomass, nitrogen, nodulation and nitrogenase activity compared with the susceptible variety Herald\(^0\). Herbicide tolerant varieties are likely to provide protection against reduced nitrogen fixation caused by Group B herbicides.

It should be possible to directly assay acetohydroxyacid synthase (AHAS) activity in living rhizobia and thus define the potential for Group B herbicides to elicit a sublethal effect on the microsymbiont. This will determine whether further effort is required to characterise rhizobia as a mode by which Group B herbicides reduce nitrogen fixation.

Recommendations

Since reductions in nitrogen fixation appear to be mainly related to the susceptibility of the plant to the applied herbicide, variety trials are potentially important sources of information pertaining to the safety of a particular herbicide by legume combination. We agree with project CSO00013 that some consideration should be given to additional work during such trials to assess the nitrogen fixation capacity. At the minimum, such trials should be carried out on legumes relying on fixation rather than soil nitrogen.

A number of factors are likely to influence whether a particular Group B herbicide will impact on nitrogen fixation of a particular legume. Climate and soil conditions contribute additional factors that would need to be considered in determining whether reduced levels of nitrogen fixation will occur. Therefore promoting a general awareness of the issues is the most appropriate response at this stage.

There is no guarantee that additional inputs of nitrogen can compensate for reduced nitrogen fixation within a legume crop
or pasture. It appears that herbicide induced reductions in nitrogen fixation are strongly linked to reduced growth of the legume. Additional nitrogen may not increase the growth of the legume since the herbicide is reducing cellular division more directly. However, application of additional fertiliser to following crops may be necessary, as nitrogen sparing or contribution from legume residues may be suppressed.

It may be necessary in the longer term to alter N calculators to account for herbicide damage to legumes, and hence reduced nitrogen sparing or contribution from legume residues.

The use of herbicide tolerant varieties such as ‘Angel’ should be a useful practical solution to herbicide induced reductions in nitrogen fixation. Since plant susceptibility to herbicide is critical for yield decline and also nitrogen fixation, selection for herbicide tolerant legumes will be a relevant strategy.

**Outcomes**

Group B herbicides have an important role in weed management strategies being used by growers across Australian broadacre agriculture. These herbicides are applied to soil or sprayed directly onto cereal crops or legume crops and pastures. Evidence exists to show that the residual herbicide present after application in previous growing seasons or in-crop applications can induce yellowing, reduced growth rates and/or decreased biological N fixation in legume crops and pastures. The project assessed the potential breadth and mechanisms responsible for these observations.

1. A screening trial was conducted to define the influence of various recommended Group B herbicides on the growth, nodulation and nitrogen fixation for the major grain legume crops and legume pasture species grown in southern Australia. For 15 of the 18 herbicide/legume combinations examined, results confirmed the negative effects on legume growth, nodulation and/or nitrogen fixation from application of the herbicide (see attachment). In eight of the 15 combinations, reductions in the amount of nitrogen fixed occurred. There was limited evidence that a reduction in nodulation and changes to plant nitrogen can occur when the rhizobia were exposed to the herbicide. It is therefore important for growers using Group B herbicides, either in-crop or in previous crops, to carefully monitor the N status to ensure optimal productivity.

2. Further mechanistic work showed that herbicides impaired root hairs and meristematic development with obvious implications for infection by rhizobia and nodule development. Reduced primary metabolism and a stress response were induced, along with reduced nitrogen assimilation likely to result in decreased nitrogen fixation activity. Plant tolerance therefore plays a critical role in whether Group B herbicides will impact on nitrogen fixation.

3. A new herbicide tolerant medic variety ‘Angel’ was compared with Herald. Plant susceptibility was confirmed as the key mechanism by which Group B herbicides impact on nitrogen fixation. Herbicide tolerant varieties will provide a practical means by which reductions in nitrogen fixation due to herbicides can be circumvented.

Group B herbicides have an important role in weed management. It is recognised that the benefits of using Group B herbicides for weed control would typically outweigh issues associated with poor N fixation in terms of dollar value. From the work completed in this project it is suggested that when Group B herbicides are used, growers should closely monitor the N status of legumes to ensure that the typical N benefits associated with legume production are attained. There is potential for variety trials to assess the susceptibility of legume varieties to suppression of nitrogen fixation by herbicides. Furthermore, consideration should be given to herbicide tolerant legumes where in-crop or residual herbicides are an issue.

**Achievements/Benefits**

Group B herbicides have gained widespread use in Australia, however their impact on nitrogen fixation by legume crops and pastures has been of concern, particularly where low rainfall and alkaline soils limit the degradation of these chemicals. These herbicides work by inhibiting the acetohydroxyacid synthase (AHAS) enzyme, in the pathway of branched chain amino acid (BCAA) synthesis. This project builds on previous work involving chickpea, which showed reductions in nitrogen fixation due to Group B herbicides, due not only to plant susceptibility, but also to interactions of herbicide with rhizobia. In this current project, a number of legume and herbicide combinations were screened to determine the scope of the problem. Root morphology and proteomics of model legume *Medicago truncatula* were examined, and a herbicide tolerant *Medicago littoralis* was studied to further elucidate the mechanisms involved. Finally an *in vivo* assay of rhizobia AHAS was developed, in an attempt to define the potential for Group B herbicides to disrupt the target enzyme in rhizobia.

The available literature on Group B herbicides and legumes was reviewed. Whilst the literature recognises legume susceptibility leading to reduced nodulation, most of the work on rhizobia was limited to *in vitro* growth assays, which did not
show any susceptibility to field concentrations of herbicide. However rhizobia with experimentally induced mutations in the BCAA pathway, including the herbicide target enzyme, are symbiotically defective, and recent work in our laboratory had shown a 'sublethal' effect of herbicide on the rhizobia leading to reductions in nodulation and nitrogen fixation. It was agreed that the initial aim of this project should be to determine the scope of Group B herbicide inhibition of nitrogen fixation, followed by a focus on the mechanisms of such an inhibition.

The first two experimental phases of the project screened a number of grain and pasture legume species with Group B herbicides recommended for weed control ‘in crop’. Field pea, faba bean, chickpea, vetch, sub clover, balansa clover, lucerne, strand medic and disc medic, along with flumetsulam#, imazethapyr# and imazamox# herbicides were screened in pot trials. These experiments were designed so that they would begin to highlight possible mechanisms by which the herbicides inhibit nitrogen fixation. They showed a range of responses, indicating that even at recommended rates of herbicide, reductions in nodulation and nitrogen fixation were related to plant susceptibility. In addition, interactions of plant applied herbicide with exposure of rhizobia to herbicide were identified.

The main message to growers from the screening work is that if Group B herbicides are applied to pasture/grain legumes or are likely to persist from application to previous cereals, nitrogen fixation by the legumes could be compromised. Under such conditions additional N fertiliser may be needed to compensate for reduced N fixation and nitrogen benefits to the following crop. The variation in the responses of individual herbicide by legume combinations meant that a set of recommendations to growers could not be made. A general awareness of the problem was seen as the outcome of this work from a practical standpoint. Results from this first phase of work were presented at the SUNFix symposium in Sydney in 2004, followed by two international meetings.

Following the screening work, the focus of the project shifted to determining the mechanisms behind reductions in nitrogen fixation. Given the complexities inherent in symbiotic nitrogen fixation, and the lack of a comprehensive understanding of the mode of action of these herbicides, a global approach was considered first. A proteomic study on the model legume M. truncatula was conducted in collaboration with the Genomic Interactions Group at the Australian National University, along with observation of root morphology of plants grown on agar. Severe changes to root hairs were observed, along with reduced relative growth rate of the primary root, and eventual loss of the primary root meristem. The formation of increased lateral root meristems was observed, indicating the loss of apical dominance concurring with shut down of the primary root meristem. However these lateral roots eventually underwent the same fate as the primary roots in this experimental system. The proteomic work identified a number of proteins whose abundance changed due to herbicide treatment. The proteins identified were generally involved in stress response and primary metabolism. Of particular interest was glutamine synthetase which is a major regulator of plant nitrogen, and a glutathione S-transferase which is involved in chemical detoxification. These results will be of interest to other researchers, and provide some clues to the herbicide mode of action, as well as the potential to disrupt nitrogen fixation.

A herbicide tolerant M. littoralis variety, FEH-1, has been developed by the South Australian Research and Development Institute (SARDI) and is now commercially available as the variety Angel. It was derived from Herald by chemical mutagenesis and screening. Angel and Herald were used in glasshouse experiments to decipher whether the impact of herbicide on nitrogen fixation was due directly to plant effects, or other aspects of the symbiosis including impacts on the rhizobia. The experiments also served as an assessment of the ability of this herbicide tolerant variety to fix nitrogen, with and without herbicide, which had not yet been studied. FEH-1 displayed tolerance to chlorsulfuron#, with greater biomass, nodulation, nitrogen and nitrogenase activity than the parent strain Herald, where the herbicide was applied to plants. However this tolerance was not complete, with a depression in the measured parameters compared with unsprayed plants. A possible penalty of the tolerance trait was evidenced by inferior performance of FEH-1 compared to Herald where herbicide was not present. Although this approach did not deliver in full the expected outcomes in terms of identifying mechanisms, valuable insight into the potential of herbicide tolerant legumes varieties to overcome herbicide limitations to nitrogen fixation was obtained, and plant susceptibility was confirmed as a major cause of depressed nitrogen fixation due to Group B herbicides.

Given that the major mechanism of reduced nitrogen fixation appears to be plant related, it remained a priority to rule out susceptibility of rhizobia to herbicide. Previous work on Group B herbicides and rhizobia had been based on growth assays in media, and one study isolated the target enzyme. The supposed 'sublethal effect' observed in our laboratory remained unexplained. An in vivo assay of rhizobial AHAS was developed with the aim of defining the potential for Group B herbicides to inhibit this enzyme in living rhizobia. The assay works by growing rhizobia in liquid culture, and applying a chemical (CPCA #
which blocks the enzyme following AHAS in the pathway for the synthesis of branch chain amino acids. This leads to an accumulation of acetolactate, the product of the AHAS enzyme. The amount of accumulated acetolactate is then colorimetrically determined. In the allocated time, the detection of acetolactate was perfected, but more work is required for optimising experimental conditions for AHAS expression and enzyme inhibition.

Other research

Further work is required to optimise an assay of AHAS activity in living rhizobia. Once complete, a screen of all Group B herbicides and inoculum strains will define the capacity for these herbicides to elicit a sublethal effect on rhizobia. The initial work would attempt to optimise the growth conditions for maximum accumulation of acetolactate. AHAS activity may be optimal at a particular stage of a broth culture. This needs to be defined. Amounts of CPCA required also need to be determined. Once this has been achieved, herbicide can be applied and changes in accumulation of acetolactate measured. The assay as currently stands uses intact, live rhizobia, which is preferable as the biological relevance of the assay is higher than other types of assays. We may find that accumulation of acetolactate does not occur sufficiently enough for reductions in activity induced by Group B herbicides to be measured. It may be necessary to concentrate cells to have enough material for the assay. The cell wall may be a barrier to herbicide, and to CPCA, the chemical which blocks the enzyme following AHAS leading to an accumulation of acetolactate. The use of a surfactant to permeabilise the cell wall may improve the assay, and will also indicate the importance of the cell wall in protecting rhizobia from ill-effects of Group B herbicides.

An important finding of this project was the potential for herbicide tolerant varieties to ameliorate the effects of Group B herbicides on nitrogen fixation. This work involved only one Group B herbicide, chlorsulfuron. Although highly informative, this experiment had two problems, in that the amount of nitrogen in the pots was higher than we would have liked, and that FEH-1 did not display complete tolerance to chlorsulfuron at the level it was applied. A second experiment using triasulfuron would be useful. FEH-1 has displayed superior tolerance to triasulfuron in field experiments. An experiment comparing the two varieties (Herald and FEH-1), three herbicide treatments (zero herbicide, sprayed on the plant, incorporated in the soil), four nitrogen treatments (mineral N, no mineral N, rhizobia, herbicide exposed rhizobia), and measuring nitrogenase activity using the acetylene reduction assay, assessing nodulation, and measuring biomass and nitrogen of root and shoot samples is proposed. This would complement the previous experiment and ensure a solid body of work for publication. If FEH-1 displays a very high level of tolerance, it is expected that any non-plant mediated effects of herbicide on nitrogen fixation will be undisputedly identified.

If selection of herbicide tolerant varieties is to be promoted as a strategy for dealing with suppression of nitrogen fixation by herbicides, their symbiotic performance should be specifically assessed. In assessing FEH-1 it was observed that in the absence of herbicide, Herald actually had higher growth, nodulation and nitrogen fixation than FEH-1. This deserves further attention.

Additional information

Additional information is also provided in the attachment to this report

An article outlining the findings of the project appeared in the April/May 2005 Grain Business (published by ABB) which reaches 18,000 grain growing operations nationwide.

The following publications were derived from this project:

2. Holmes et al 2006 J. Proteome Res. 5:2309-2316.