New genetic opportunities from legume intercrops for controlling Striga spp. parasitic weeds

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Abstract

In smallholder farming in East Africa, intercropping of maize with the cattle forage legume, Desmodium uncinatum Jacq., prevents parasitism by Striga hermonthica (Del.) Benth. (witchweed) through an allelopathic mechanism. Isoschaftoside, a di-C-glycosylflavone, isolated from the root extract and root exudate of Desmodium, interferes with in vitro radicle development of germinated Striga. The biosynthetic pathway of this class of compound is already mostly present in edible legumes and in cereals, so characterisation of the enzyme and genes that control C-glycosylflavone biosynthesis has the potential to create this protection mechanism in other agriculturally important plants.

Keywords: Desmodium uncinatum; Striga hermonthica; C-glycosylflavone; C-glycosyltransferase; Zea mays; allelopathy; weed control; isoschaftoside

1 INTRODUCTION

Witchweeds, comprising parasitic plants in the Striga genus, also commonly called striga, are a major threat to the staple food crops of over 100 million people in Africa.1 They germinate close to their host plants in response to specific chemical signals from the latter, of which the hydroquinone (sorgoleone)2–4 and sesquiterpene lactones, especially strigolactones,5–7 are typical. The radicle subsequently grows and, when approaching the host root cells, undergoes haustoriogenesis giving rise to the functional attachment organ through which parasitism is initiated. This review describes how the agricultural practice of intercropping Desmodium with maize in Striga-infested areas suppresses parasitism of maize and the authors’ investigations into the mechanism of suppression. The state of research into the allelopathic mechanism by which chemical agents mediate this process, which is known to work in the field, is explained, along with how these discoveries may be exploited in the future as studies on the biology and chemistry of the interaction move forward and offer molecular biological opportunities.

2 STRIGA INHIBITION BY DESMODIUM IN THE FIELD

Intercropping maize with cattle forage legumes comprising Desmodium spp. [silverleaf D. uncinatum (Jacq.) (Fabaceae) and greenleaf D. intortum (Mill.)] reduces dramatically the infestation of maize and sorghum by Striga hermonthica (Del.) Benth. and is similarly effective against S. asiatica (L.) Kuntze.8,9 As a consequence of this, not only is the maize yield increased but soil fertility is improved by nitrogen fixation and reduced soil erosion.10 Further examination of the mechanism by which Desmodium prevents Striga parasitism on maize was conducted. Field plots were established with maize intercropped with D. uncinatum with and without added nitrogen fertiliser, maize monocrop with and without added nitrogen fertiliser and maize monocrop with artificial ground covering of maize stover with and without added nitrogen fertiliser (Fig. 1).8 These data show that Desmodium is able to reduce Striga parasitism through ground shading (mimicked by stover covering) and also through nitrogen fixation (as shown by the experiments that added fertiliser), but significantly greater reduction of Striga parasitism by Desmodium cannot be explained by these physical and soil fertility factors alone, indicating an incremental effect through an allelopathic mechanism in addition to contributions from the other factors.

The allelochemical-mediated effect was confirmed in pot experiments. Desmodium uncinatum plants were grown in pots on shelves, and water dripping from their root system was used to irrigate maize planted in Striga-infested soil. Desmodium was planted with and without Rhizobium inoculation, to compare the effect of fixed nitrogen and the source of root chemistry. Comparisons were made between maize plants grown in Striga-infested soil irrigated by water from pots containing Desmodium

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Figure 1. Effects of nitrogen, shading and Desmodium uncinatum intercrop on numbers of Striga hermonthica plants parasitising maize in field plots, Mbita Point Field Station, Kenya, 1999–2000, short rains. Different letters are significantly different (P < 0.5) by Tukey’s studentised range test. Data are means of six replicates.

Table 1. Suppression of Striga hermonthica infestation of maize plants grown in pots at various levels of nitrogen, with or without Desmodium uncinatum and Rhizobium spp. (3000 seeds pot⁻¹, 50 replicates)

<table>
<thead>
<tr>
<th>Nitrogen (kg ha⁻¹)</th>
<th>Maize + D. uncinatum + Rhizobium spp.</th>
<th>Maize + D. uncinatum</th>
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<td>120</td>
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<td>172</td>
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Table 1. Suppression of Striga hermonthica infestation of maize plants grown in pots at various levels of nitrogen, with or without Desmodium uncinatum and Rhizobium spp. (3000 seeds pot⁻¹, 50 replicates)

with and without Rhizobium spp. and water passed through autoclaved soil. Results showed a clear suppression of parasitism by Desmodium root exudates but with no apparent effect from Rhizobium spp. (Table 1), verifying that the source of biological activity was the Desmodium plant.⁹

Efforts to elucidate the chemical ecological mechanism underpinning this phenomenon have been pursued. Desmodium root chemistry has been studied using both root extracts and root exudates. Extracts are obtained by macerating root tissue in a suitable solvent (depending on the polarity of small molecules of interest) and has the advantage of being easy to do on a large scale, even from field-grown plants, but it contains unwanted bulk, and purification of individual components is more difficult. Root exudates are obtained by hydroponically growing plants in a suitable medium and then transferring them to distilled water for a collection period during which the organic material is exuded.¹¹ Although this technique produces very small quantities of material and is difficult to do on a large scale, it provides an ecologically relevant metabolic profile and gives a guide to the quantities available in the environment that could confer allelopathic properties.¹²

The phytochemical profiles of Desmodium root exudates and root extracts reveal a complex array of plant secondary compounds. Bioassays have shown that components of the profile are responsible both for germination stimulation and for subsequent radicle growth inhibition.¹¹ A combination of the two effects provides the hypothesis for the allelopathic mechanism by which Desmodium prevents parasitism and continuously removes Striga seed bank from the soil in situ.¹³ Thus, Striga germination is stimulated by Desmodium and maize root exudates, and it is the subsequent development of Striga that is disrupted, as demonstrated by in vitro bioassays showing radicle growth inhibition. However, radicle growth inhibition may not be the most important effect Desmodium causes, and the physiological details of the exact mechanism are currently being investigated. This hypothesis is consistent with the finding that, in an experimental intercrop involving maize and D. uncinatum in a Striga-infested area at Mbita Point in western Kenya, the parasitic weed seed bank was virtually depleted in 7 years (12 seeds kg⁻¹ soil), unlike in maize monocrops or maize-food legume intercrops where increases in the seed bank occurred (over 800 seeds kg⁻¹ soil).¹³

In practice, when the intercropping system is first employed on the farm, rows of maize (75 cm apart) are intercropped with Desmodium (also 75 cm apart). In the first year, plots are hand weeded early in plot establishment and again after 5 weeks. After maize is harvested, the Desmodium is either left to produce seed, currently a valuable crop with demand for seed, or is cut back to leave stubble and used as forage. Desmodium is perennial, and so in subsequent seasons it is cut back for forage before maize is planted and again clipped after 3 and 6 weeks along with hand weeding of the plot. The Desmodium intercrop is only acceptable in smallholder farming practice as there is economic benefit in it. The sale or use of seed or cut Desmodium as a nutritious nitrogen-rich
cattle forage for stall-fed cattle has allowed an increase in dairy cow numbers, with individual farmers able to afford a dairy cow for the first time. The nutritional content of the forage legume has allowed ‘exotic’ breeds of high-milk-yielding cows to be introduced into the area, improving the nutritional status of the population and income generation.14,15

3 DESMODIUM ROOT CHEMISTRY IS THE SOURCE OF THE ALLELOPATHIC MECHANISM

Compounds were isolated for the first time from the biologically active root exudates and root extracts of Desmodium by bioassay-guided fractionation and HPLC purification. Initial bioassays using crude root exudates of Desmodium showed no inhibition of Striga germination, and so subsequent bioassays were performed to measure Striga germination and the length of the developing radicle. The phytochemical profile is very complex, but all the active compounds so far isolated are from the flavonoid/isoflavonoid pathways. The authors’ examination of the metabolome of Desmodium root extracts and root exudates has identified biologically active isoflavonones such as uncinanone A, B (germination stimulant) and C (radicle inhibitor), as well as other isoflavones and pterocarps, initially isolated from fractions that stimulated germination but which proved not to be active compounds.11,16

Radicle inhibition was further investigated by bioassay-guided fractionation of the more polar fractions of the root metabolome that were known in their crude state to possess more radicle inhibitory activity.11 A compound was isolated that showed radicle growth inhibition at a low concentration (1 ppm). The structure of this sample was analysed by electrospray mass spectroscopy (ESMS), permethylation EIMS17 and microprobe NMR spectroscopy, and the structure was elucidated as the di-C-glycosylflavone 6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranosylapigenin, also known as isoschaftoside (Fig. 2).

The authors have since discovered further C-glycosylflavones present in the polar fractions of Desmodium root extracts; bioassays of radicle length inhibition are near completion and show that other C-glycosylflavones possess some activity in vitro assays. So far, the C-glycosylflavones of Desmodium all possess an 8-C-glucopyranosyl moiety (Fig. 2). One of these structurally related compounds is 8-C-β-D-glucopyranosylapigenin (vitexin).

However, although this product from a single glycosylation inhibits radicle length, it does not show the same potential to prevent parasitism in bioassay. The analysis of the root exudates from hydroponically grown Desmodium revealed only one of these C-glycosylflavones to be present to a marked degree, namely isoschaftoside, the compound previously found to be biologically active for radicle length inhibition and implicated in interfering with Striga development. The concentration of purified C-glycosylflavones that show biological activity must be compared with the concentrations found in root exudates from hydroponics or soil to ascertain whether the material is active at ecologically relevant concentrations.

Food legumes such as cowpea, beans and soybean share the flavonoid/isoflavonoid metabolic pathways with Desmodium and can stimulate the germination of Striga, but they demonstrate no significant post-germination allelopathic effects on Striga.18 It is hypothesised that the germination stimulation effect can be mediated by a large number of structurally similar aromatic moieties from the flavonoid/isoflavonoid pathways, as well as by any strigolactones present.5 However, the biosynthesis of isoschaftoside, which is key to the mechanism of parasitism prevention, requires specific C-glycosyltransferase (CGT) enzymes that convert precursors present in all these plants to the highly active post-germination inhibitors.

Figure 2. C-Glycosylflavones characterised from the roots of D. uncinatum.
4 PLANT C-GLYCOSYLTRANSFERASES (CGTS)

C-Glycosylflavones are known in a taxonomically diverse range of terrestrial plants, with over 100 structures reported from 54 angiosperm families at the time of a 1981 review, and possess a range of biological activities. They have been reported as cucumber phytoalexins and mycorrhizal colonisation stimulants of melon root. The C-glycosylflavone maysin is a host-plant resistance factor against the corn earworm, Helicoverpa zea (Boddie), and fall armyworm, Spodoptera frugiperda, in Zea mays L.

Previous biosynthesis experiments proposed that C-glycosylation is not a terminal step in C-glycosylflavonoid biosynthesis but must occur at a stage earlier than flavone production (Fig. 3) at which point only O-glycosylation is observed. The flavanone naringenin, an earlier biosynthetic intermediate in flavone biosynthesis, has been shown to be 8-C-glycosylated to vitexin in Spirodela polyrhiza (L.) Schleiden (although these experiments did not rule out the possible conversion of naringenin to 2-hydroxynaringenin before C-glycosylation). Another C-glycosyltransferase was isolated from buckwheat, Fagopyrum esculentum Moench (Polygonaceae), cotyledons. This 41 kDa protein was purified and was not specific to glycosylating reagents and could utilise UDP-glucose and adenosine diphosphoglucose (ADP-glucose), as well as UDP-galactose and UDP-xylose to a lesser extent. However, it was specific in glycosylating 2-hydroxynaringenin and would not glycosylate flavones or flavanones. In this case, 2-hydroxynaringenin could be converted to vitexin and isovitexin (Fig. 3). The production of two C-glycosylated products can be explained by the ring opening of 2-hydroxynaringenin to a symmetric glycosyl acceptor. In the case of D. uncinatum, only 8-C-glycosylflavonoid molecules are found, which suggests a non-symmetrical intermediate for glycosylation. Therefore, it is likely that naringenin or 2-hydroxynaringenin is the glycosyl acceptor and two CGTs are required for the biosynthesis of isoschaftoside in D. uncinatum, although one enzyme may be able to utilise both UDP-glucose and UDP-arabinose. However, the regiochemistry of the C-glycosylflavone may ultimately not be due to the glycosylation step, as the regioisomers of glycosylated 2-hydroxynaranginones continue to be in equilibrium, but due to the final dehydration step that fixes the conformation. This may be a spontaneous chemical dehydration, which occurs quickly in acidic conditions, or the result of a specific dehydratase enzyme. The authors have not found the intermediary mono-C-glycosylflavone 6-C-α-L-arabinosylapigenin (isosmollupentin) in D. uncinatum, which suggests that the glycosylations are sequential, with glucose introduction before arabinose.

Although the isolation of C-glycosylflavones has often been reported in planta in the natural products literature, there are almost no data available about the CGT enzymes responsible for the process. No plant gene sequences have been annotated for a CGT, and no amino acid sequence for a plant CGT protein has ever been completed, in spite of early work in which one

**Figure 3.** Putative biosynthetic pathway for the production of C-glycosylflavones in planta and the allelochemical isoschaftoside in Desmodium uncinatum. CHS, chalcone isomerase; IFS, isoflavone synthase; DH, dehydratase; CGT, C-glycosyltransferase; FNS II, flavone synthase II.
was purified from plant soluble protein mixtures and shown to be active in vitro. Success in characterising a CGT will be the first known example in plants. Although the vast diversity of plant secondary metabolite substrates and the many possible sites of reaction (including N, S, O and C atoms) have yet to generate a specific sequence annotated as a CGT, there are many annotated O-glycosyltransferases (OGTs), and many proposed to be involved in O-glycosylation of flavonoid metabolites. This suggests that there may be no specific domain to distinguish CGTs from OGTs, making an approach from identification of candidate genes unlikely to succeed. It is hypothesised that the specificity shown for C- rather than O-glycosylation lies with the substrate binding site, and plant genes annotated as OGTs may indeed code for a CGT. The authors therefore propose a classical protein purification approach to obtain plant CGTs from Desmodium or a gene-sequenced plant, specific for the glycosylation of intermediates in the biosynthesis of isoschaftoside, and subsequently to obtain genetic information from the protein sequence.

5 BIOTECHNOLOGICAL EXPLOITATION OF ALLELOPATHIC DESMODIUM ROOT BIOCHEMISTRY

Many smallholder subsistence farmers in Kenya would prefer intercropping maize with edible crop legumes rather than cattle-fodder legumes. In particular there is an immediate demand for legumes such as cowpea (Vigna unguiculata L.) or beans (Phaseolus vulgaris L.). Neither has demonstrated potent suppression of Striga.18 Now that some understanding has been gained of the secondary metabolism involved in the mechanism by which Desmodium suppresses Striga, there is the potential for transferring CGT biochemical traits into edible legumes. Reports of C-glycosylflavones in pearl millet, wheat and maize, plants that are parasitised by Striga, have not analysed the root chemistry or root exudate chemistry, and for any compound to be effective it must be produced in the correct ecologically relevant environment, and at a physiologically effective concentration. Legumes possess relevant chemistry in the root tissue, as ecological interactions with Rhizobium are mediated through flavonoids and their exudation into the environment.

5.1 Breeding biochemical pathways for Striga-controlling allelochemicals into food legumes

Traditional breeding between Desmodium and other food legumes may not be successful. However, food legumes already possess the biosynthetic pathway for flavone biosynthesis, which comprises most of the desired biochemical pathway to C-glycosylflavones. It is possible that specific CGT genes conferring the glycosylation required to generate isoschaftoside are present and either not expressed or expressed at very low levels. Screening a range of edible legume species and cultivars to examine their metabolome should be completed, including legumes that show resistance to the legume-specific Striga gesnerioides (Willd.), for identification of plants that may already possess combinations of the biosynthetic pathway elements for producing C-glycosylflavones. Of particular interest is the presence of any mono-C-glycosylflavones and naringenin or 2-hydroxynaringenin. These plants would be candidates for breeding programmes to construct the complete mechanism for allelopathic Striga control.

5.2 Exploiting the post-germination inhibition of Striga development in Desmodium

While isoschaftoside has been identified and shown to be biologically active, characterisation of the root exudate metabolome has not been completed and may yield more allelopathic chemical targets. This process is facilitated by the ease of hydroponically growing Desmodium, and, although the authors’ studies are based on this method, hairy root cultures have also been created that possess the same root extract chemistry rich in C-glycosylflavones (Hamill JD, unpublished).

The C-glycosylflavones implicated in the protection mechanism are biosynthesised by a branch of the flavonoid biosynthetic pathway separate from action by the legume isoflavone synthase (IFS) and prior to flavone formation (Fig. 3). The direct biosynthetic precursors are yet to be determined for Desmodium. However, previous research suggests28,29 that 2-hydroxynaringenin is the precursor, with glycosyl-UDP or glycosyl-ADP acting as the glycosyl donor. 2-Hydroxynaringenin is the intermediate in the conversion of flavanones to flavones. In the case of the Apiaceae, this transformation is controlled by a 2-oxoglutarate-dependent dioxygenase (FNS I), and the 2-hydroxyflavonone intermediate is not produced.30 However, in legumes, this transformation is controlled by a NADPH-dependent cytochrome P450 monoxygenase (FNS II). There are two types of FNS II reported. The enzyme identified from Gerbera hybrida (CYP93B2) generates the flavone directly from the flavanone,31 but, in the case of Glycyrrhiza echinata (L.) (CYP93B1)32 and Medicago truncatula (Gaertn.) (CYP93B10, CYP93B11),33 the enzyme product is the 2-hydroxyflavonan, which suggests that this intermediate may be available in the legume metabolome for biosynthetic transformation without the requirement to introduce a 2-hydroxyflavanone synthase. Biosynthetic incorporation studies using root proteins are already under way to determine the precise biochemical pathway and intermediates that are substrates for glycosyl donors. Efforts to identify a Desmodium CGT are also in progress. Once found, other CGT sequences may be identified by homology screening in edible legumes, or by location through genome sequences should the biosynthetic pathway enzymes cluster. Alternatively, genetic transformation of the CGT into other phaseolus species, including cowpea, for which technology already exists, is a realistic goal.

5.3 Locating CGTs in genome-sequenced legumes or crop plants

In the case of sequenced legumes, particularly Lotus japonicus (lotus), Medicago truncatula (medicago) and Vigna unguiculata (cowpea), finding suitable metabolome chemistry could be used to help determine the function of annotated glycosyltransferases. In addition, homologous genes to the CGT identified from Desmodium can be cloned and functionally expressed to identify if they possess the correct chemoselectivity. Clustering of biosynthetic pathway genes may provide a clue to the function of homologous glycosyltransferases. Once characterised, these genes would be available for further transformation into edible legumes with complementary biochemical pathways to produce the protection mechanism.

The transfer of these genes directly into cereals can also be contemplated. Vitexin is present in pearl millet (Pennisetum spp.)34 and in finger millet (Eleusine coracana (L.) Gaertn.)35 and 6-C-glycosylflavones such as isovitexin and isoorientin are present in wheat (Triticum aestivum L.),36 where the use of herbicide safeners increases CGT activity, while other processes reduce the
overall C-glycosylflavone content. Root-specific promoters may be required to facilitate this approach in cereals. Isolation of CGT activity through the genome again is made difficult by the inability at present to propose a putative enzyme through sequence information, even differentiating between O-, C-, S- or N-glycosyltransferases. Biosynthesis experiments with intermediates and glycosyl donors would be required to show functional expression of native or transformed CGT proteins. Again, screening of particular cultivars expressing CGT activity and the biosynthesis of suitable C-glycosylflavonones would allow traditional breeding to generate the allelochemicals rather than through genetic modification.

6 CONCLUSION

It has been discovered that the C-glycosylflavone isoschaftoside, present in Desmodium root exudate suppressing Striga parasitism, is biologically active in interfering with Striga development. Whether it alone can be instrumental in suppressing Striga parasitism is currently being investigated. The impact of intercropping maize with Desmodium and exploiting this allelochemical effect in sub-Saharan Africa on over 6000 farms has alleviated 50 000 people in Kenya, Uganda and Tanzania from the food insecurity caused by parasitic weeds.37 This is an example of using allelopathy in a significant role for weed control in an economically important arable system, and elucidating the mechanism will demonstrate the potential for extending this concept in creating pest management strategies for weed control in the developed world. The biochemical pathway for isoschaftoside production in Desmodium is being studied and shows potential for transfer into edible crop legumes, as all but one or two biosynthesis enzymes are expected to be present in most legumes and key CGT activity may be present in others.

The possible impacts to human health in the production of C-glycosylflavonones in human edible legumes must be considered. C-Glycosylflavonones are already present in many plants in the human diet and are associated with health foods for their antioxidative properties implicated in prevention of cancer and heart disease in the developed world.38 However, in higher quantities, along with flavones such as apigenin and luteolin, they possess antithyroid effects,34 particularly when part of a staple food, as this increases the quantities consumed. As well as monitoring the concentrations of these compounds in root exudates and in root tissues, the concentration in edible parts of the plant should be examined, too, in order to determine their suitability. Discovery of Striga suppression was serendipitous, as the choice of intercropping plants was originally designed to inhibit insect attack by various species of stemborer on maize, for which the system is also effective.39 There is no ecological explanation yet as to why this process has evolved, as S. hemonthica does not parasitise legumes. However, S. gesnerioides does attack legumes, and the effect of Desmodium on S. gesnerioides is now being studied. Full elucidation of the mechanism by which Desmodium controls Striga in the field is vital to ensure quality assurance of intercrop seeds that possess the required chemistry and to determine the way in which evolution of resistance may occur. Host-plant selectivity by Striga and its germination stimulation by Desmodium is a subject that requires more investigation, especially with regard to structures of strigolactones and other germination stimulation through specific host root chemistry.5–7

This intercropping strategy is especially suitable in the field conditions of East Africa. The transfer or identification of this protection mechanism in other intercrop cereals, especially human edible ones, will extend the range and increase the economic benefits. At present it is providing benefits to the very poorest smallholder farmers in East Africa, but it could form part of integrated strategies for farm practices in more affluent areas. Although the control of Striga through seed bank reduction is unlikely to be effective as a single control measure, as only a fraction of the seed bank responds to host-plant stimuli in a particular season, Desmodium may be useful in a suicidal germination strategy. The development of more highly effective or species-specific germination stimulants,7–9 either chemically applied or generated in situ by plants and used in conjunction with Desmodium, would allow treatment in the presence of crop plants and increase the depletion rate of seed banks, as the increased levels of germinated Striga could not attach to the host plant. Many of the proposed and realised methods of genetic transformations or plant breeding of resistance to ameliorate damage caused by Striga parasitism will eventually cause Striga to evolve resistance.40 There is a large genetic diversity in the Striga seed bank, and protection mechanisms may also select for resistant or increased virulence in strains of parasitic weed.41 The integration of intercropping adds another mode of action that can reduce selective pressure to slow the evolution of resistance and protect the genes selected for or introduced into crop plants.

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