Tannin accumulation, biochemistry and genetics in the genus *Medicago*

A report prepared for Graham Butcher of Rural Solutions Gore

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Executive summary

This report summarises the potential for the development of cultivars of *Medicago* (including Lucerne – *Medicago sativa*) that are capable of accumulating tannins in their leaves. Accumulation of leaf tannins in forage is a powerful tool for the reduction of bloat in ruminant livestock that are fed readily fermentable feed.

Medicago (barrel medic) is an important pasture legume with a range of physiological features that makes it highly desirable for dryland agricultural situations (Brummer et al., 2009; Hawkins and Yu, 2018; Gholami et al., 2014; Waghorn et al., 1998). However unlike the related Genus *Lotus*, *Medicago* species lack the ability to accumulate leaf tannins. *Medicago* does, however, retain full biosynthetic capacity for the accumulation of tannins in seeds. This indicates that while they have the *potential* to accumulate tannins in various tissues, *Medicago* species lack the ability to activate this pathway in leaves. This point is important as it points to a regulatory block rather than a lack of biosynthetic ability to produce tannins, the latter being a much more difficult situation to overcome.

The lack of ability to accumulate leaf tannins has been reported to extend across all species in the genus (Goplen et al., 1980). Thus traditional breeding approaches to generate lines accumulating tannins to high levels in leaves has failed likely due to the lack of genetic and phenotypic variability within populations with which to breed. Opportunities to reach outside of the genus for the required genetic traits via tools such as wide species hybridization or indeed cross genera hybridization (between *Medicago* and *Lotus* for example) are fraught and to date have failed to bring the requisite genetics for leaf tannin production into *Medicago*.

A significant body of research has been carried out to determine the biological reasons for the inability to 'switch-on' tannin biosynthesis in leaves in *Medicago* (Li et al., 2016b; Dixon et al., 2013; Verdier et al., 2012a; Xue et al., 2012). This research has necessarily focused on the use of transgenic approaches to alter the pattern of expression of the key regulators of tannin biosynthesis in *Medicago*. In other words what molecular switches need to be activated in leaves to enable the accumulation of tannins? This has proven to be a highly successful strategy and lines have been produced (and the technology patented; Verdier et al. (2015), US Patent 9,121,031) that accumulate industrially significant levels of tannins in the leaves of the engineered plants. While very promising this is of little use to NZ farmers due to the current regulations governing the release and use of GMOs in this country.

Specifically the reported changes are focused on engineering the regulation of 2 specific transcriptional activation proteins such that they are expressed in leaves leading to the coordinated activation of the tannin biosynthetic pathway in leaves. Such gain-of-function mutations rarely occur in nature and likewise are rare occurrences in many commonly used mutation breeding approaches (which mostly produce loss-of-function mutations).

To date this has been the end of investigation in New Zealand to find non-transgenic means to address this issue in Lucerne. There are however two potential approaches to introduce genetic variation that may lead to new varietals of *Medicago* that are able to accumulate leaf tannins. These include somatic hybridization of cells from *Medicago* and *Lotus,* or the use of naturally occurring mutagens that are known to lead to gain-of-function mutations at high rates – transposons.

The use of somatic hybridization to introgress new alleles/genetics from *Lotus* to *Medicago* is an unlikely route with reports of somatic hybridization between reproductively isolated members of *Medicago* species (Bingham et al., 2013; Mendis et al., 1991). This does not mean that the approach is not a valid one however, but requires substantial effort to recover any viable plants. There is only

one report where somatic hybridization has been successfully reported between *Lotus corniculatus* and *Medicago sativa* (Niizeki, 2001).

The use of transposons (Mobile genetic elements), to generate pools of mutated plants is a valid approach and, in terms of the biosynthetic pathway responsible for the production of tannin precursors, has been successful in numbers of plant species (Butelli et al., 2012; Fernandez et al., 2013; Walker et al., 2007; Fernandez et al., 2010). Indeed the use of a tobacco transposon inserted into *Medicago truncatula* (generating a transgenic population of plants) has produced a valuable resource for genetic studies in this model legume (D'Erfurth et al., 2003a; Tadege et al., 2008; Cheng et al., 2013). However the use of native elements to achieve genetic variation is new, but is gaining significant interest from the international community as a valid breeding strategy (Paszkowski, 2015). My team at Lincoln University has pioneered this approach in grapes and latterly in Hops. We are proposing to investigate the feasibility of this approach in *Medicago* as a scoping exercise to determine the level of effort that would be required to identify plants with the rare mutations required to deliver tannin production in leaves.

Conclusions:

1. There is very little opportunity to use traditional breeding approaches to generate *Medicago* varieties that accumulate tannin in their leaves.
2. Transgenic approaches are the best way forward and have been successfully trialled and will be available to the market within 24 months
    a. NZ farmers will ***not*** be able to use this material as it is Genetically Modified and under existing legislation and the current political climate it is highly unlikely that the current situation will change. While there is a pathway to commercial release of GMOs the costs (in the millions of dollars) far outweigh the benefits and given the current political climate it is the authors view that any such application is bound to fail.
    b. The best outcome for a legislative change would be a de-emphasis on the process (i.e. the methods for producing GMOs) and emphasise the regulation of the application and assess risk based on this. In other words if the application has sufficient benefit and low risk then it should be allowed to be released.
3. The molecular switch required to produce leaf tannins is relatively simple therefore alternate approaches to GMOs are possible
    a. Somatic hybridization between Lotus and *Medicago* is possible but requires a very large and expensive effort. Work to date has not yielded desired traits.
    b. The use of naturally occurring transposons is a route that in nature delivers the phenotypes we are interested in. However this approach is untested in *Medicago* and represents a high risk approach
4. We are proposing to carry out a highly focused and targeted proof of concept project to investigate the feasibility of the transposon approach to yielding new varieties of *Medicago* that accumulate leaf tannins.

Introduction:

Condensed tannin formation in forage legumes is an important component for the control of bloat in grazing ruminants, sheep and cattle in particular (Dixon et al., 2013; Kumar et al., 2018; Goplen et al., 1980). *Medicago* species, and in particular *Medicago sativa,* are important forage legumes in the New Zealand context (Waghorn et al., 1998). However when used as a stock feed *Medicago* species, often contribute to bloat due to the fact that they don't accumulate tannins that would otherwise contribute to limiting bloat in grazing animals.

Tannins – a component of flavonoid biosynthesis

Condensed tannins are a group of polymerised polyphenolic compounds. Precursors of condensed tannins are produced by the action of the phenylpropanoid pathway and the flavonoid/anthocyanin pathway in particular. Like many complex metabolic pathways there are complex regulatory mechanisms that act in concert to deliver the particular end-points (for example, pigmented anthocyanins, flavonoids - the non-pigmented UVB protectants and condensed tannin precursors) in the cells of particular tissues at specific developmental times. So a single pathway can therefore be directed to certain outcomes by altering the pattern of expression of individual genes – not unlike the shunting yard in a railroad assembling trains to be delivered to different locations.

Medicago is able to produce condensed tannins

All species of the Medicago genus so far tested are incapable of production of condensed tannins in their ***leaves*** (Goplen et al., 1980). However they are capable of the production and accumulation of condensed tannins in the seed coat (Verdier et al., 2012a). This indicates that *Medicago* possess and in-tact and functional biosynthetic pathway for tannin accumulation, but not the molecular switch-gear to turn on tannin formation in vegetative tissues. The key regulator of tannin biosynthesis in *Medicago* has been identified (MtPAR) and have been shown to be able to activate tannin accumulation in tissues other than seed coat but does not lead to levels of tannin accumulation sufficient for production against bloat (20mg/g dry weight) (Verdier et al., 2012a).

Two potential routes to accumulation of leaf tannins in Medicago

There are two main routes to produce *Medicago* varietals that accumulate tannins in leaves, traditional breeding or genetic modification/engineering. For a traditional breeding approach to be successful there has to be sufficient variation in leaf based tannin accumulation available within any given species or within the genus. From early studies, measuring the presence of leaf tannins across a wide range of *Medicago* species, it was found that none of the species in this genus produced significant levels of leaf tannin (Goplen et al., 1980). This fact underpins the lack of success for breeding for increased leaf tannins in economically important species of this genus.

The lack of genetic diversity leaves researchers with three pathways forward, two technically very challenging and the other fraught with regulatory issues. Within the wider group of forage legumes (the *Galegoids*), the *Robinoid* family, which include *Lotus*, are capable of accumulation of relevant levels of leaf tannins (Waghorn et al., 1998). Transfer of the requisite genetic and allelic variation from lotus to *Medicago* cannot be achieved via traditional breeding. However very wide species hybridization from the tertiary gene pool (e.g. *Lotus*) to the primary/target gene pool (e.g. *Medicago sativa*) might be possible via forced somatic hybridization (Niizeki, 2001). However as will be explained later this approach is made very difficult due to the difference in the chromosome number and overall lack of sequence similarity between *Lotus* (1n=6-7) and *Medicago* (1n=7-8) making the production of viable hybrid offspring difficult.

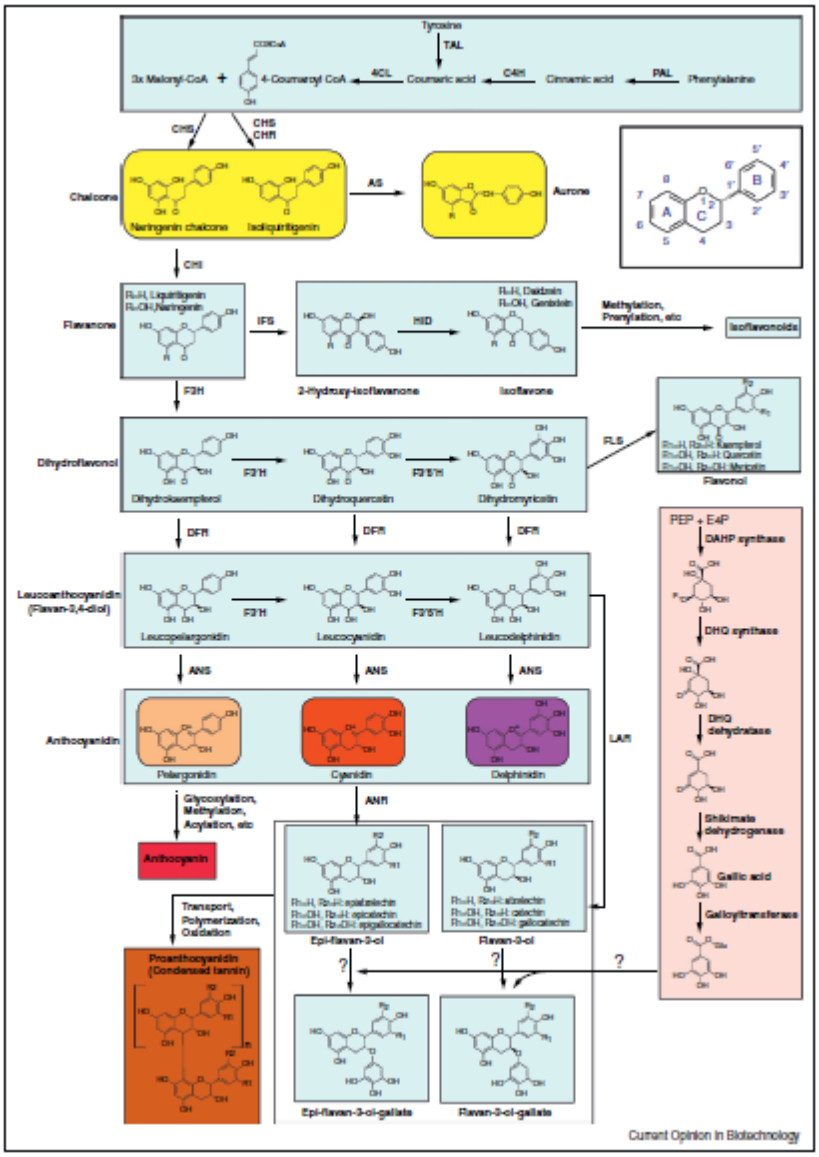
A second approach is to utilise naturally occurring mobile genetic elements within the *Medicago* genome (called transposons) to generate a wide field of genetic variation that might lead to the upregulation of key regulatory genes such as MtPAR in leaves. Transposons are present in every branch of life and have been implicated as a major contributor to evolution (Lisch, 2013). A number of international groups, including our team at Lincoln University, have been attempting to determine how to activate these elements in a range of crop species to generate new genetic and phenotypic variation (Lanciano et al., 2017; Cho et al., 2019; Thieme et al., 2017). The activation of transcriptional regulators by virtue of insertion of a transposable element insertion into or near the promoter of the gene has been shown to occur in a number of crops such as Grape and Orange (Butelli et al., 2012; Fernandez et al., 2010). Such an insertion in the MtPAR gene might be able to alter patterns of expression of this important regulator of tannin formation such that leaf accumulation of tannins is possible

The final approach is the use of transgenic/genetic modification to introduce a new copies of MtPAR and MtLAP1 with leaf specific promoters into *Medicago* species thus enabling the activation of tannin biosynthesis in leaves. This approach has been patented by members of the Sam Nobel foundation in the USA, led by Prof. Rick Dixon (US Patents: 7,709,701 & 9,121,031), and anecdotally *Medicago* varietals with this modification are progressing through regulatory approvals in the USA prior to release to the market in the near term. These materials through proven are unavailable for NZ farmers due to the regulatory difficulties with release from containment of transgenic/genetically modified organisms.

In this report I will briefly describe the biochemical pathway leading to proanthocyanidin accumulation, the molecular regulation of this pathway and possible mechanisms to overcome the lack of genetic diversity for this trait in the *Medicago* genus. I will finish by describing a number of experimental approaches by which we might consider to circumvent these genetic blocks so to realise the introduction of genetics into this plant that does not involve genetic modification.

Proanthocyanidin biosynthesis

Condensed tannin formation is derived from the phenylpropanoid and flavonoid biosynthetic pathways (see Figure 1). These pathways contributes to a range of important secondary metabolites, in particular the precursors of lignin, the isoflavonoids, the pigmented anthocyanins and the colourless flavonoids. Condensed tannin precursors can be produced via the action of two specific enzymes Banuyls (BAN)/Anthocyanidin Reductase (ANR), Xie et al., 2003), and Lucoanthocyanidin reductase (LAR; Tanner et al., 2003). The catechin and epichacitin precursors of the condensed tannin polymer are monomeric and polymerization of these subunits into the tannin molecular occurs via the action of laccase type enzymes,(Pourcel et al., 2005), and in a variety of different tissues (e.g. the testa of seeds, the seed coat and leaves or other tissues).



Scheme for the biosynthesis of flavonoids, with emphasis on anthocyanins and condensed tannins. The numbering scheme for the majority of flavonoids described in the present article is shown in the box, second line right. Abbreviations for enzymes are: ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; AS, aurone synthase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; 4CL, 4-coumarate coenzyme A ligase; CHR,

Figure 1. The flavonoid and tannin biosynthetic pathway. Taken from Dixon et al., (2013)

Molecular regulation of condensed tannin formation

As previously mentioned condensed tannins can be produced by *Medicago*, however their formation appears largely restricted to the seed coat (Dixon et al., 2013). This is important as it clearly indicates that all of the requisite enzymatic machinery is present and functional in *Medicago*. What is lacking, however, is the molecular switch in leaf tissue that would activate the biosynthetic pathway and enable the accumulation of these compounds in leaves.

Extensive work has been carried out by a number of groups internationally to describe in detail the molecular regulation of this pathway. In particular work carried out by researchers at the Sam Noble Foundation in the USA, have described a small number of key regulators that appear critical for the formation of condensed tanning in plant tissues and in particular in *Medicago truncatula*. These regulators are MtPAR and MtLAR, both being MYB class transcriptional regulators (Verdier et al., 2012b, 2012a, Li et al., 2016b, 2016a).

Regulation of the flavonoid pathway is characterised by a tripartite interaction between three families of transcriptional regulators; the MYB class of transcriptional regulators, the bHLH class of transcriptional regulators and the WD40 class of transcriptional regulator. Tissue specific, coordinated expression of these three classes of proteins allows tissue specific and branch specific expression of the pathway leading to deposition of the endpoints of this pathway being tightly regulated (Liu et al., 2015).

Various groups have shown that in manipulating the location and timing of expression of these factors can influence the location of production of condensed tannins. This work has thus formed the basis for introduction of leaf tannin formation in *Medicago*, highlighting the power of GMO approaches to circumvent genetic blocks such as this (Verdier et al., 2012b, 2012a, Li et al., 2016b, 2016a; Lei et al., 2017; Dixon et al., 2013).

While a genetic modification route is the most obvious pathway forward for the introduction of leaf based condensed tannins in *Medicago*, in the New Zealand context this approach is not viable. While in theory the legislation would allow us to develop such lines in containment in the laboratory, the costs and restrictions associated with even seeking a limited release or restricted field trial essentially present a complete ban to release of such material and therefore commercial use on farm.

Newer approaches of genetic modification that would not see introduction of foreign genetic material, such as the much vaunted gene-editing technology CRIPR-Cas9, are not considered to be genetic modifications in jurisdictions such as the USA, Canada, China and Japan. However these editing approaches have been recently banned in both Europe and NZ.

As I will highlight below the options for circumventing this regulatory issue to generate the genetic variation required to introduce tannin accumulation in leaves are time consuming, labour intensive work arounds that are far less targeted than genetic modification approaches currently being pursued in the USA, predominately by the Sam Nobel Foundation.

Thus while “bloat-safe” *Medicago* lines are being produced and commercially tested at the moment in the USA, it is quite certain that for the time being NZ farmers will not be able to avail themselves of these latest advances.

New (old) approaches to introduce the genetics for tannin accumulation in leaves of *Medicago* species.

As mentioned above the current regulatory environment leaves the NZ pastoral industry in a difficult situation. There is insufficient genetic variation in this trait for conventional breeding programmes to introduce leaf based tannin accumulation. The most sensible approach (genetic modification) is banned, leaving us with only a limited number of work arounds, that are largely untested and expensive. Anecdotally the situation is so dire that obvious science providers (such as AgResearch), deem this problem so difficult that it cannot/should not be solved in New Zealand (Graham Butcher; Pers. Comms).

With this in mind there are however two potential approaches than can be taken. One quite old yet potentially powerful; somatic hybridization/cybrid formation between *Lotus* (which does accumulate tannins in its leaves, and *Medicago,* and a new approach utilising naturally occurring genetic elements that are proposed to be potent agents in plant evolution; Transposons.

Somatic Hybridization

Somatic hybridization represent a form of wide species (in this case Genera) hybridization and recovery of plants. In essence we are seeking to hybridize the primary gene pool (*Medicago*) with a member of the tertiary gene pool (*Lotus*), and in doing so fuse the two genomes and recover plants with a blend of each. This would provide a hybrid that may be a potential source of intermediate breeding material to introgress the necessary genetic material to deliver leaf based tannins from *Lotus* into *Medicago*.

The process basically involves tissue culture of both species, followed by the physical fusion of cells to facilitate the hybridization of the genomes. This is then followed by recovery of plants from these cultures and analysis to determine the breadth of the hybridization. Major sticking points in this approach are that it is highly likely that recovery of fertile progeny will be limited and that the genetic fusions generated will be very complicated. Thus the recovery of the exact genetic combinations we desire are also likely to occur at low frequency. If we are successful in recovery of fertile plants it is also certain that we would then have to carry out numerous rounds of crossing back to the *Medicago* parent to recover the commercially viable offspring. While this approach appears quite daunting, it ***has*** been successfully attempted and plants have been recovered highlighting this as a viable approach (Niizeki, 2001). While a preliminary set of experiments is being planned in my group at Lincoln University, it is clear that even if we are successful in recovering plants, this attempt will be little more than a proof of concept due to the daunting amount of time and resource required to deliver a commercial product.

Transposons

Hidden in the genomes of all of all organisms are a collection of mobile genetic elements called transposons. They are thought to be captured remnants of viral invaders of cell that have largely lost the ability to leave the cell in which they have found themselves other than through propagation of the host. Typically these elements make up a large proportion of our genomes (50% of the Human genome and up to 90% of plant genomes). While they have been variously referred to as parasitic and “junk-DNA” recent advances in genomics reveals a much more intriguing role for these genomic hitch-hikers in the evolution of species, in particular when populations come under rapid changes in environmental conditions (Negi et al., 2016; Lisch, 2013; Vitte et al., 2014; Feschotte and Pritham, 2007; Fedoroff, 2012; Negi et al., 2016).

Transposon mobility in genomes can impact function of genes in a number of ways. The most obvious is their insertion into genes causing their inactivation. However there are a number of other important genomic interactions that can result from transposon mobility including gain-of-function mutations whereby insertion of a transposable element into the controlling region of a transcriptional activator turns of expression of an entire pathway in a new tissue type (Lisch, 2013). A particularly poignant example of this is the natural mutation leading to the formation of blood Oragnes (see Figure 2). In this example a transposon has landed upstream of a MYB transcription factor leading to the cold temperature driven accumulation of pigments (anthocyanins) in the flesh of the orange (Butelli et al., 2012). This is the exact type of mutation we would require in *Medicago* to deliver leaf based tannin accumulation.

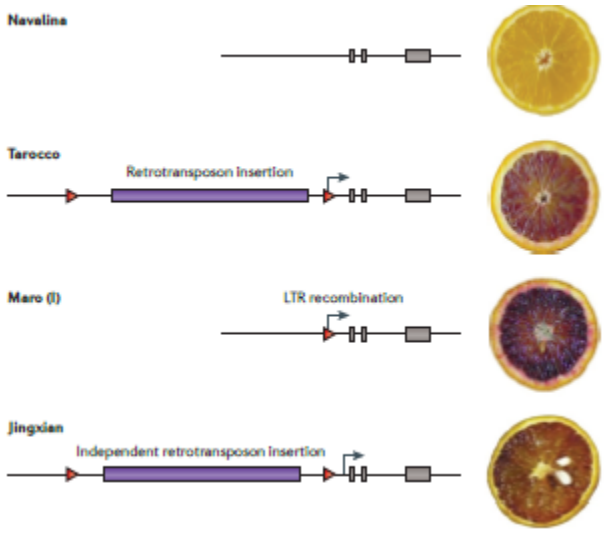


Figure 2. Different varieties of blood oranges have variants of the same retrotransposon inserted into the promoter region of the MYB transcription factor RUBY. The presence of the transposon confers cold stimulated expression of the anthocyanin pathway in the flesh of blood oranges. The RUBY MYB transcription factor is a master regulator of this pathway. Figure taken from Lisch, (2013)

My team at Lincoln University has been exploring the possibility of accelerating transposon activity in a number of crops species (Grape, Hop and Potato). We have successfully shown it to be possible to launch transposons in these genomes and are currently seeking to increase the levels of activation to usable levels. We have produced a population of grapevines and are embarking on the same for both Hops and potatoes. We are aware of a similar approach in *Medicago*, where a GM approach has been taken to introduce a transposon from tobacco, *Tnt-1*, that has successfully produced a population of plants which is used extensively for gene by function studies (Iantcheva et al., 2009; D'Erfurth et al., 2003b; Cheng et al., 2013; D'Erfurth et al., 2003a; Tadege et al., 2008; Sun et al., 2019). This work clearly shows that activation of transposons is capable in *Medicago*, however the type and extent of activation of native elements is currently unknown. We are seeking to take a very targeted and small scale approach to test this by activation of native transposons in *Medicago* cell cultures and screening of these cell cultures for the ability to produce condensed tannins through staining of the cells with a tannin specific dye DMCA (Abeynayake et al., 2011).

A rapid, simple and cost effective method for qualitative measurement of tannins

Finally both novel genetic approaches outlined above require a functional screen that is cost effective and fast that allows for high throughput testing of individual cells or plants for the accumulation of condensed tannins. Current methods for the measurements of tannins and/or their precursors often involve the use of High Performance Liquid Chromatography coupled to high-end Mass Spectrometry detectors (HPLC-MSMS). While clearly the gold standard for analytical analysis of the tannin/tannin precursors accumulating in plant tissues, they are neither cheap nor necessarily high throughput.

Tannin precursors are readily detectable using the dye DMCA (see Figure 3), having been successfully used to detect tannin precursor accumulation in individual cells and as a semi quantitative measure of accumulation (Abeynayake et al., 2011; Glavnik et al., 2009). We are currently working these methodologies into a test kit that will both serve as a rapid qualitative screening tool and as a potentially costs effective tool for the measurement of tannin accumulation in Lotus in response to cultivar and environment.

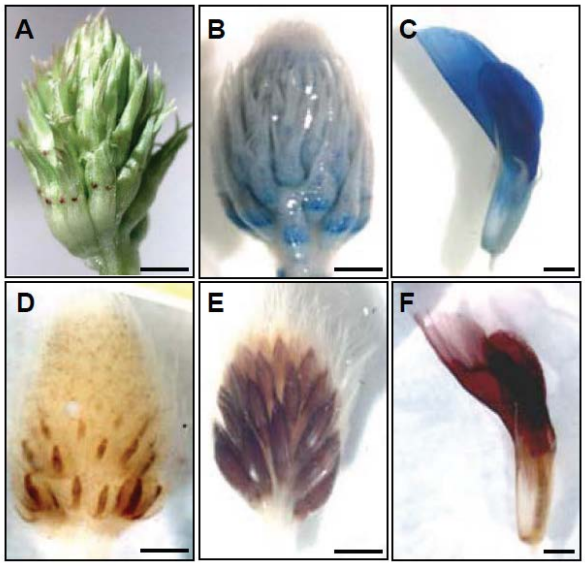


Figure 3. An example of DMACA staining of clover florets. An immature inflorescence before (A) and after (B) DMACA staining. A mature flower after DMACA staining (C). Immature inflorescences at early (D) and late (E) stages of development and a mature flower (F) after DMACA staining and embedding in LR White resin. Bars represent 2 mm. Figure taken from (Abeynayake et al., 2011)

Summary

Tannin accumulation in *Medicago* leaves cannot be conventionally bred for. However tannins do accumulate in the seeds of these species. Genetic modification approaches seeking to alter the regulation of the biosynthetic pathway are in existence, the approach being patented and plant are currently being filed tested for imminent release in the USA. These materials are unable to be used by NZ farmers due to the current anti-GM regulatory regime. Few options exist to develop such materials in NZ due to costs and the lack of desire of research providers to pursue such avenues of research in NZ.

There are however two pathways that could be pursued that have a good likelihood of success, given financial support and time. These being somatic hybridization between *Lotus* and *Medicago* (a very long term project with many complications) and the use of transposons to accelerate the accumulation of new and novel genetic diversity in *Medicago* (a untested approach in these species, though not without precedent).

The Winefield group at Lincoln University is embarking on a a number of small focused proof-of-concept experiments to judge the relative opportunities for each approach as well as developing a tool set for the rapid detection and quantification of tannin precursors in plant tissues.

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