



# Sustainable Management of *Medicago sativa* for Future Climates: Insect Pests, Endophytes and Multitrophic Interactions in a Complex Environment

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*Medicago sativa* L. (alfalfa, syn. lucerne) is an important forage crop for livestock, which is subject to attack from a range of insect pests and susceptible to diseases that can reduce production and persistence. This review considers the main insect pests affecting *M. sativa* in China and New Zealand as well as the wider plant resistance mechanisms and multitrophic interaction that occur between plants, insect pests, entomopathogens, endophytes, the environment, and climate change. This is with a view to identifying new research opportunities applicable to *M. sativa* that can be applied to improving production and persistence of this important agricultural crop. These opportunities include identification and activity of entomopathogens/endophytes (e.g., *Bacillus* and *Pseudomonas* spp., *Metarhizium* spp.) and plant growth enhancers (*Trichoderma*), as well as multitrophic plant-insect-microbial interactions.

**Keywords:** alfalfa, insect pests, multitrophic interactions, biocontrol, plant resistance mechanisms, endophytes, rhizosphere, climate-change

## INTRODUCTION

*Medicago sativa* L. (alfalfa, syn. lucerne) is an important perennial leguminous forage worldwide (Michaud et al., 1988; Huyghe, 2003; Lamb et al., 2006; Veronesi et al., 2010; Annicchiarico et al., 2015). The widespread use of *M. sativa* is due to its high adaptability for growth in a range of environments, particularly under drought conditions (Annicchiarico, 2007; Huang et al., 2018), its ability to form symbiosis with rhizobium and biologically fix nitrogen (Carlsson and Huss-Danell, 2003), its high protein value (Vance et al., 1979; Ruckle et al., 2017), and forage yield potential, either under grazing or “cut and carry” cropping regimens (Small, 1996). It can be grown with both temperate and tropical grasses, or as a standalone crop (Capstaff and Miller, 2018), across a range of climates. Nitrogen fixation by rhizobia associated with roots not only provides substantial amounts of nitrogen to plants and soil, but reduces the need for artificial nitrogen fertilizers (Carlsson and Huss-Danell, 2003). In many cases, there is a positive correlation between nitrogen fixation and legume dry matter yields (Carlsson and Huss-Danell, 2003).

In November 2019, a bilateral workshop between AgResearch (New Zealand) and the Institute of Plant Protection-China Academy of Agricultural Science (IPP-CAAS, China), was held to

discuss opportunities to cooperatively address challenges in agricultural production common to both countries. Critical challenges to agriculture and *M. sativa* in particular, were the growing impact of abiotic and biotic threats on insect pests, biocontrol, beneficial microbes (e.g. entomopathogens, soil microbes), abiotic environmental stresses on *M. sativa*, and the current and future state of knowledge around these aspects. This review examines how these aspects influence plant growth, persistence, and quality in general with a focus on *M. sativa* where appropriate. In particular, the trophic interactions, that have been observed in *M. sativa* and other plants species are discussed with a view of establishing areas for future research.

In China, *M. sativa* is grown for livestock and poultry, with the area sown continuing to expand and demand higher than current supply. The plant is considered integral to the transformation of traditional agriculture (Zhang et al., 2018; Xu et al., 2021). It also provides an important alternative to overgrazing of ecologically sensitive grassland environments (Zhang et al., 2018; McNeill et al., 2021). *Medicago sativa* is seen as an important crop in the development of animal husbandry, increasing farmers' income, and promoting social and economic development in rural areas (Li, 2019; Xu et al., 2021). The most recently available data show that in 2017, 4.15 million ha<sup>2</sup> of alfalfa was being grown with a total yield of 29.3 M tons (National Animal Husbandry Service, 2017). In New Zealand, *M. sativa* has been promoted as a suitable forage species for New Zealand dryland systems for over 100 years (Moot, 2012). It is considered an important dryland species for grazing and stored winter forage and is particularly valuable to farmers in environments where traditional ryegrass-white clover plant species cannot persist (Avery et al., 2008; Moot, 2012). It is also grown under irrigation as a forage grazing crop for dairy cows (Smith, 2015). *Medicago sativa* in New Zealand is planted either as a monoculture, destined to be harvested for stored winter feed and, to a lesser extent, for feed pellet production, or when utilized for grazing, either as a monoculture or in combination with a grass species, often tall fescue, *Festuca arundinacea* Schreb. (Poales: Poaceae). The plant is grown across 200,000 ha producing approximately 2.4 M tons annually and additionally fixes 30 kg N<sub>2</sub>/ton of legume grown. Animal production from *M. sativa* yields approximately 700 kg of red meat per ha (D. Moot, Lincoln University, pers. communication). Primary production areas outside China and New Zealand include the United States, Canada, Italy, France, south Russia, Argentina, Chile, South Africa and Australia (Yuegao et al., 2009).

## MAJOR INSECT PESTS OF *M. SATIVA*

Worldwide, a range of insect pests (and plant parasitic nematodes and diseases) are known to attack *M. sativa*, resulting in yield reduction, loss of persistence of stands and plant quality (Leath et al., 1988; Manglitz and Ratcliffe, 1988; Compendium of Alfalfa Diseases Pests, 2016). Losses occur both above- and below-ground and are affected directly through feeding on the foliage or roots, as the result of insect-mediated virus transmission (e.g., aphids) or the ingress of pathogens through wounds caused

by insects (Godfrey et al., 1986). The main pests in China are aphids and thrips, followed by alfalfa weevil [*Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae)]. Aphids and thrips are the most widespread taxa throughout the regions where *M. sativa* is grown, while *H. postica* is a significant pest in the Ningxia and Xinjiang regions. Other pests include *Heliothis virescens* (Hufnagel) (= *Heliothis dipsacea*) (Lepidoptera: Noctuidae) (He et al., 1997) and the beet webworm (*Loxostege* spp. L.) (Zhang et al., 2005a). Several species of aphids are damaging to *M. sativa*, with *Acyrtosiphon kondoi* (Shinji et Kondo), *Aphis craccivora* (Koch), *Acyrtosiphon pisum* (Harris) and *Therioaphis trifolii* (Monell) being the main pest species. Similarly, several species of thrips attack the crop with the major pest being *Odontothrips loti* (Haliday). *Thrips tabaci* Lindeman, *Frankliniella occidentalis* (Perg.) and *Frankliniella intonsa* (Trybom) also cause damage. Aphids and thrips reduce both the yield and nutritional value of *M. sativa* (Zhang et al., 2005b, 2017; Wu et al., 2013) and act as vectors of viral plant diseases. Aphids (Garran and Gibbs, 1982; Roumagnac et al., 2015) and thrips (Li J. et al., 2021) have been reported to be carriers of alfalfa mosaic virus and alfalfa leaf curl virus. As the area of *M. sativa* cultivation increases, so too does the incidence and impact of insect pests and viruses (Wang et al., 2021), seriously restricting further development of the industry. Based on 2017 data, it was estimated that *M. sativa* pests cause at least 20% yield loss, with an average direct economic loss of 9.144B ¥ p.a. (2.03B NZD p.a.; 1.44B USD) (Li et al., 2020).

In New Zealand, insect pests are a persistent and significant economic cost to grassland and forage production systems (Zydenbos et al., 2011; Jackson et al., 2012; Ferguson et al., 2019), with a few key pests having a major impact on production and longevity of *M. sativa*. These key species are all exotic (i.e., non-native to New Zealand) and comprise *Sitona discoideus* Gyllenhal, three aphid species (spotted alfalfa aphid (*Therioaphis maculata* (Buckton)), blue green aphid (*Acyrtosiphon kondoi* Shinji), and *A. pisum*). Lesser pests are white fringed weevil [*Naupactus leucoloma* (Boheman)] and little fringed weevil [*Atrichonotus taeniatus* (Berg)]. Like many *Sitona* species, *Sitona discoideus* causes both above-ground damage from adult feeding on foliage, and below ground damage to firstly the nitrogen-fixing root nodules and subsequently the root system (Goldson et al., 1988), resulting in both short- and long-term reduction in yields (Goldson et al., 1985; Goldson and Muscroft-Taylor, 1988). Prior to the introduction of biological control agents, high populations of aphids caused death of seedlings and significant yield losses in established stands (Kain and Trought, 1982; Cameron et al., 1983). The introduction and establishment of parasitoid biological control agents as part of classical biocontrol programmes was effective in controlling *S. discoideus* (Goldson et al., 1990) and the three aphid species (Cameron and Walker, 1989), along with the releases of aphid resistant cultivars (Kain and Trought, 1982).

## ENDOPHYTES

### Bacterial Endophytes

Bacteria belonging to the 'root-colonizing rhizosphere-competent bacteria' including members of the genera

*Pseudomonas* (e.g. *P. fluorescens*), *Azospirillum* (e.g. *A. brasilense*), and *Bacillus* are often also found as colonizers of the internal tissue of plants (Hardoim et al., 2008). Root nodules of leguminous plants have been found to host large population of endophytic bacteria of diverse genera and species which are unrelated to rhizobial symbiotic nitrogen fixing bacteria (Zakhia et al., 2004; Muresu et al., 2008). Stajković et al. (2009) found that co-inoculation of non-rhizobial strains with *Sinorhizobium meliloti* positively influenced *M. sativa* nodule numbers, but significant effects on growth parameters with respect to inoculation with *S. meliloti* alone were absent. However, single inoculation with other non-rhizobial strains caused significant increase in shoot and root parameters compared to uninoculated plants, indicating that non-rhizobial strains possess plant growth promoting potential.

Research in China has identified endophytic fungi (*Rhizoctonia solani*, *Trichoderma harzianum* and *T. atrovirideum*) and bacterial endophytes (*Bacillus* and *Pseudomonas* species) in *M. sativa*. The bacterial endophytes have been found to be active against the root rot pathogen *Fusarium oxysporum* F. sp. *Medicaginis* (Chen et al., 2020). However, there is a paucity of research to demonstrate whether these fungi have other benefits to *M. sativa*. Conversely, endophyte metabolites may also benefit plant pests. For example, the presence of a *Bacillus* strain isolated from *M. sativa* seed was shown to improve the longevity of the free-living nematode *Caenorhabditis elegans* (Maupas) (Zhang et al., 2018).

Knowledge of bacterial endophytes with demonstrated insecticidal effects is much less developed. Perhaps the most prominent example is *Bacillus thuringiensis* (Lacey et al., 2015), one of the few bacterial endophytes deployed as a commercial biocontrol agent against *Lepidoptera*. Another example is *Brevibacillus laterosporus* (Laubach) Shida et al., 1996, originally isolated from surface-sterilized cabbage leaves (Ormskirk et al., 2019). Two *Bacillus laterosporus* Laubach strains have been shown to exhibit pathogenicity against selected *Lepidoptera*, *Coleoptera*, *Diptera*, and *nematodes*, as well as antimicrobial activity especially against bacteria and fungi (De Oliveira et al., 2004; Ruij, 2013). Of particular interest are two strains isolated in New Zealand that cause mortality against larvae of the diamondback moth (*Plutella xylostella* L.) (van Zijl de Jong et al., 2016). Although *B. laterosporus* has not yet been found in *M. sativa*, other endophytic bacteria belonging to the genus *Brevibacillus* were isolated and exhibited a positive effect on the number of *M. sativa* nodules while increasing shoot and root parameters (Stajković et al., 2009).

## Fungal Endophytes

Fungal entomopathogens (*Beauveria* spp.), often solely considered as entomopathogens, have been shown to play additional roles in controlling plant pathogens, plant growth promoters and beneficial rhizosphere colonizers (Jaber and Ownley, 2018; Michaud et al., 2018, references therein). Entomopathogens such as *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) and *Lecanicillium* spp. (formerly *Verticillium lecanii*) (Hypocreales: Cordycipitaceae), have also been shown or implicated in having antagonistic

activity against both insect pests (Lewis and Cossentine, 1986; Cherry et al., 2004) and plant pathogens (Clark et al., 2006). As such, these fungi present opportunities for multiple use of in integrated pest management strategies (Summers, 1998).

## INTERACTIONS BETWEEN THE PLANT AND ITS BIOTIC PARTNERS: *M. SATIVA* AS A HOLOBIONT

### Plant-Insect Interactions

The relationship between plants and their insect herbivores has a long evolutionary history (Labandeira, 2013), and one that has been described as an evolutionary “arms race” between plants and herbivorous insects. In response to insect attack, plants have developed physical and chemical defenses to prevent or mitigate feeding and/or oviposition (Trumble et al., 1993; Scharidl et al., 2013; Meiners, 2015; Zhang and Li, 2019). Physical defenses include slippery or sticky plants surfaces, trichomes, waxy cuticles, hardness, and architecture to deter feeding (Small, 1996; Whitney and Federle, 2013). Plant chemical protection includes plant secondary defenses that are inducible, and not only protect the plant from UV-light, desiccation or cold, but also from insect herbivores, fungi, bacteria, and viruses (Hartmann, 1996). *Medicago sativa* possess both biochemical (Agrell et al., 2003; Julier et al., 2004; Wu et al., 2021) and physical defenses (Lovinger et al., 2000) against insect pests. However, the relationship is not often explored beyond the plant-pest level and ignores the relationship to abiotic (soil fertility, moisture, heat stress) and biotic (soil microbiome, endophytes) variables. Tritrophic interactions between plants, insect herbivores, and their natural enemies provides another level of complexity to plant defenses and act to mitigate the impacts of insect herbivores, or biocontrol agents. Herbivore-induced plant volatiles (HIPVs) play a key role in these interactions, as they can attract insect predators and parasitoids to herbivore-attacked plants (Turlings and Erb, 2018). This signaling occurs both above and below ground, and herbivore-damaged roots shown to produce volatiles that attract entomopathogenic nematodes (Rasmann et al., 2005).

More recently, it has been shown that the soil microbiome may also play an important role in plant defenses against above- and below-ground insect herbivory (Howard et al., 2020). Pineda et al. (2019) demonstrated that the composition of the soil microbiome reduced pupation of thrips, *Frankliniella occidentalis* (Pergande), placed on chrysanthemum leaf cuttings. Conversely, above ground herbivory has been shown to alter the soil microbiome, but the effects were conditional on plant type, soil-microbial community, soil fertility and temporal changes (French et al., 2021; Sveen et al., 2021).

### Insect Response to Secondary Metabolites

In response to plant secondary compounds, insects have evolved strategies to overcome these defenses including the ability to suppress or avoid plant stress responses, including suppression of herbivore induced plant volatiles (HIPVs) (Turlings and Erb, 2018). Endosymbionts, specialized bacteria found in insects—are important in allowing the insect to utilize otherwise



unsuitable host plants by enabling digestion of plant polymers, detoxification of plant-produced toxins, provisioning of essential nutrients, and providing protection from parasitoids and pathogens (Frago et al., 2012; Hansen and Moran, 2014; Oliver and Martinez, 2014). Endosymbionts have also been shown to protect their insect hosts from abiotic stress (Guo et al., 2017; Lemoine et al., 2020).

## Trichoderma

*Trichoderma* (Ascomycetes, *Hypocreales*) spp. are cosmopolitan free-living fungi found in foliar environments, soil, fungal material, decaying wood and sediment (Kubicek et al., 2003; Harman et al., 2004; Jaklitsch and Voglmayr, 2015). Their value to the host plant is protection from fungal pathogens, increased plant nutrient uptake, solubilization of soil nutrients, and induction of systemic resistance (Harman et al., 2004; Guzmán-Guzmán et al., 2019). Secretion of effector proteins and secondary metabolites ( $\beta$ -glucanases, harzianolide) by *Trichoderma* are understood to mediate the beneficial interaction between *Trichoderma* and plants (Guzmán-Guzmán et al., 2019). Defensive interactions have also been indicated at both the biochemical and molecular level, the defense response potentially fluctuating between induced systemic resistance (ISR) and systemic acquired resistance (SAR), depending on the *Trichoderma* strain and plant species, as well as biotic and abiotic conditions (Nawrocka and Ma.olepsza, 2013). A small pot experiment looking at the effect of *Trichoderma harzianum* Rifai on *M. sativa* growth with and without cutting, found significant increases in plant shoot and root dry weights of mowed plants treated with *T. harzianum*, compared with the control unmown plants treated with *T. harzianum* (Zhang et al., 2019). Soil available nutrients (N, P, K) were also elevated compared to the control. More recent research has highlighted the presence of mycovirus in *Trichoderma* (Lee et al., 2017; Wu et al., 2020). Mycoviruses have been shown to exhibit greater genetic diversity and host range than previously thought, with the ability to move easily between fungal hosts belonging to unrelated (distant) taxonomic groups (Nerva et al., 2017). Although the role of mycovirus in *Trichoderma* remains unclear, it has been shown that infected *Trichoderma* can mediate antifungal activity against plant pathogens (Chun et al., 2020).

## Plant-Pathogen Interactions

Plants have also been shown to exhibit defensive priming, whereby stimuli from pathogens, beneficial microbes or arthropods, can lead to enhanced activation of induced defense mechanisms throughout the plant. When the plant is subsequently challenged, there is a faster and/or stronger reaction to attack (Maleck and Dietrich, 1999; Mauch-Mani et al., 2017). This adaptive strategy improves the defensive capacity of plants and can involve physiological changes, increased transcriptome and metabolic levels, and epigenetically modulated changes in gene expression. Upon subsequent challenge, the plant effectively mounts a faster and/or stronger defense response that results in increased resistance and/or stress tolerance (Mauch-Mani et al., 2017). Perhaps not surprisingly, plant defense responses can be complex, with both antagonistic and co-ordinated interactions

shown to occur between defensive pathways (Maleck and Dietrich, 1999), as well as trade-offs between growth and defense functions (Bastias et al., 2021).

## Plant-Endophyte Interactions

The term 'endophyte' describes (micro)organisms living inside plant tissues including roots, leaves, stems, flowers, and seeds (Pinski et al., 2019), and can be both bacteria and fungi. In a strict sense, an endophyte must not be detrimental to its host plant (see Lodewyckx et al., 2002 for definitions), but the plant receives an ecological benefit from the presence of the symbiont (Quispel, 1992). In most cases, these benefits include better germination (Ulloa-Muñoz et al., 2020), mobilization of nutrients (Khalifa et al., 2016), and antagonistic effects toward phytopathogens (Pinski et al., 2019; Whitaker and Bakker, 2019).

While research on endophyte bioactivity has focussed on fungi (Arnold et al., 2003; Strobel, 2018), bacterial endophytes, especially those with entomopathogenic activity, are an emerging research area. This is against a backdrop of an increasing failure of conventional agricultural practices (Wemheuer et al., 2016; Le Cocq et al., 2017) and pest management systems (Lewis et al., 1997; Summers, 1998) to provide sustainable food production systems.

A more novel utilization of endophytes is in bioprospecting to identify biologically active compounds with antibiotic, antioxidant, antiparasitic, or cytotoxic activities, or the facilitation of reactive dye discoloration, where endophytes are used as the main produces rather than the plant itself (Savi et al., 2019). For a more in-depth overview on the concept of endophytism and the various beneficial interactions between endophytes and their host-plants and their impact on agriculture and, ultimately, human health, refer to the comprehensive review by Khare et al. (2018).

Both bacterial and fungal endophytes have been isolated from *M. sativa* (Hardoim et al., 2008; López et al., 2018; Chen et al., 2020), but there is a paucity of research that has examined the stability and prevalence of these endophytes in the plant. For example, one of problems for *M. sativa* cultivation is growth under drought and salinity stress (Noori et al., 2018). Bacterial endophytes related to *Klebsiella*, *Kosakonia*, and *Sinorhizobium* found in *M. sativa* root nodules have demonstrated a positive effect on resistance to salinity stress, and provide the potential to extend the arable climate zones for *M. sativa*. *Halomonas* and *Bacillus* endophytes isolated from plants that are capable of growth in high salinity, have been shown to colonize *M. sativa* and enable seedling growth in the presence of up to 1% NaCl, a non-permissible salt concentration for non-inoculated plants (Kearl et al., 2019).

## Plant-Endophyte-Large Herbivore Interactions

Induced responses in plants, initiated by herbivory, create potential for trait-mediated indirect interactions among herbivores, with saliva shown to alter plant response to feeding, which may benefit plant pathogens or insect herbivores. Research by Liu et al. (2016) on the transcriptome response of two-week-old *M. sativa* seedlings following application of

cow saliva to cut leaves, indicated negative effects on regrowth, as well as modification of jasmonic acid synthesis, potentially enhancing the susceptibility to pathogens. Artificial cutting of perennial ryegrass (*Lolium perenne* L.) leaves induced synthesis of an *Epichloë* endophyte alkaloid, which reduced feeding by adult Argentine stem weevil [*Listronotus bonariensis* (Kuschel)]. However, this effect was mitigated by sheep (*Ovis aries* L.) saliva applied to the cut sections (Bultman et al., 2018). The authors speculated that the observed effect may involve salivary enzymes or perhaps even metabolites from living microbes within the saliva. Moose (*Alces alces* L.) browsing on Scots pine (*Pinus sylvestris* L.) were found to improve the performance of the sawfly (*Neodiprion sertifer* Geoffroy) compared to unbrowsed trees (Nordkvist et al., 2019). Interestingly, the compound di-terpene, known to affect sawfly performance, was not affected by the browsing treatments nor correlated with sawfly performance parameters, suggesting other secondary compounds were active in the response.

Plant secondary compounds can also have direct impacts on large herbivores. Coumestrol, a phytoestrogen compound is naturally found in *M. sativa*, and if elevated in the leaves, can have detrimental impacts on sheep reproductivity (Fields et al., 2018). Foliar fungal infection or aphid feeding have both been shown to increase the concentration of coumestrol, sometimes to levels that could interfere with ewe reproductive performance (Kain and Biggs, 1980; Purves et al., 1981; Fields et al., 2018). Toxins associated with endophyte (*Epichloë* species; Clavicipitaceae) commonly associated with cool season grasses (Poaceae, subfamily Poöideae), not only have activity against insects but also vertebrates (Johnson et al., 2013; Schardl et al., 2013; Hume et al., 2016). However, whether bacterial endophytes associated with *M. sativa* have an impact on large herbivores is unknown.

## Multitrophic Interactions

Tri-trophic relationships have also been shown to be intricate in terms of plant signaling and concomitant response from natural enemies. A study investigating plant host effect and parasitism by the endoparasitoid *Diaeretiella rapae* (M'Intosh) on cabbage aphid, *Brevicoryne brassicae* (L.) genotypes, found that plant cultivar had significant effect on aphid reproduction, rates of parasitism and parasitoid sex ratio (Mehrpavar et al., 2019). As *B. brassicae* lacks facultative endosymbionts that may provide protection from parasitism (Clark et al., 2012), the effect on *D. rapae* reported by Mehrpavar et al. (2019) cannot be attributed to known defensive endosymbionts. However, as found with *A. pisum*, the effect of host plant on the incidence of the defensive endosymbiont *Hamiltonella defensa*, and associated parasitism rates can be minimal (Sochard et al., 2019; Smith et al., 2021). Insect herbivore-induced plant defenses are well-known to influence the abilities of baculoviruses to infect, replicate in, and kill their insect hosts (Ali et al., 1998; Shikano et al., 2017). *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidiae) caterpillars treated with a sublethal dose of baculovirus and feeding on tomato (*Solanum lycopersicum* L.) plants, elicited the highest plant anti-herbivore defense compared to plant responses to healthy caterpillars (Pan et al., 2019). However, it was not

known if the increase in plant defenses induced by virus-infected caterpillars benefited the virus or the caterpillar (Pan et al., 2019). The use of volatiles that attract natural insect pest enemies has been suggested as a way of controlling important agricultural pests through a push-pull strategy (Pickett and Khan, 2016) and has been successfully used in maize against lepidopteron stem borers (Pickett et al., 2014). Increases in plant peroxidase (POD) and catalase (CAT) activities, along with salicylic acid (SA) concentration, have been suggested as an explanation for enhanced defense response to *A. pisum* in *M. sativa* inoculated with the mycorrhizal fungi *Rhizophagus intraradices* (N.C.Schenck & G.S.Sm.) C.Walker & A.Schüssler (Li et al., 2019).

## FACTORS IMPACTING THESE INTERACTIONS

### Climate Change

While the environmental impacts of climate change are complex (Pedrono et al., 2016; Yang et al., 2021), current and future predictions indicate that crop losses associated with insect pests, plant pathogens, and weeds, will become more frequent and substantial (Baker et al., 2015; Deutsch et al., 2018; Anderson et al., 2020). This includes shifts in insect distribution (Battisti and Larsson, 2015; Ricciardi et al., 2021), loss of biodiversity (Bellard et al., 2014), and associated impacts on ecosystem services (Pedrono et al., 2016). There will also be increased biosecurity impacts as pathways and vectors associated with trade and tourism provide the means to move high impact pests and diseases, rapidly and across vast distances (McNeill et al., 2021; Ricciardi et al., 2021).

The effects of climate change, particularly elevated CO<sub>2</sub> and temperature, and water availability on *M. sativa* production and persistence, has been and continues to be an area of significant research. Research has demonstrated both positive and negative impacts on photosynthesis, respiration, nodulation function and efficiency, biomass allocation, growth and quality of *M. sativa*, which are covered in the reviews by Liang et al. (2013), Aranjuelo et al. (2014), and Soares et al. (2019).

Elevated CO<sub>2</sub> levels will also change insect phenology and their impact parameters. High carbon:nitrogen (C:N) ratios associated with increased CO<sub>2</sub> levels have been shown to affect the performance of insect herbivores positively (aphids) but to be mostly neutral for chewing insects (Whittaker, 1999). Aphids grown at high CO<sub>2</sub> levels were found to be more responsive to aphid alarm pheromones, potentially making them more vulnerable to parasitism (Awmack et al., 1997). Elevated CO<sub>2</sub> was also shown to increase the duration of nymphal stages, decrease adult longevity and fecundity of *A. pisum* (Li C. et al., 2021). The researchers also found changes in the nutritional profiles of the aphid, with a significant interaction between CO<sub>2</sub> levels, and nymph generations.

A study on above- and below-ground herbivory on white clover (*T. repens* L.) by clover root weevil (*Sitona obsoletus*, (Gmelin), formerly *S. lepidus*) found that elevated CO<sub>2</sub> resulted in significantly increased root C:N ratios, increases in root mass

(85% greater) and nodule abundance (220% more abundant) (Johnson and McNicol, 2010). Adult *S. obsoletus* consumed significantly more foliage but laid fewer eggs under elevated CO<sub>2</sub>. Conversely, there was increased larval abundance and performance at elevated CO<sub>2</sub>, which was positively correlated with the number of nodules available (Johnson and McNicol, 2010). The authors concluded that reduced foliar quality at elevated CO<sub>2</sub> was generally disadvantageous for adult *S. obsoletus* living above-ground, but very beneficial for larvae feeding on the root system, due to enhanced nodulation (Johnson and McNicol, 2010). Research to determine if this response is found with other *Sitona* species known to feed on *M. sativa* (e.g., *S. discoideus* Gyllenhal, *S. hispidulus* F.) has yet to be undertaken.

Climate change may impact the seasonal ecology of parasitoids, with consequences on host–parasitoid synchrony and population cycles, food-web functioning, and ecosystem services such as biological pest control (Jamieson et al., 2012; Tougeron et al., 2020). Research on the effect of abiotic and biotic factors on larch casebearer [*Coleophora laricella*, (Hübner)] defoliation of larch (*Larix* spp.) and its two parasitoids *Agathis pumila* (Ratzeburg) and *Chrysocharis laricinellae* (Ratzeburg), found that warming temperatures disrupted the complex interaction between trophic levels possibly contributing to casebearer outbreaks in North America (Ward et al., 2020). The broad concepts discussed above for both above- and below- ground interactions are shown in **Figure 1**.

## Genotype

Despite the evident importance of endophytes–bacterial and fungal–little is known about the genetic background that drives the evolution of the plant–endophyte–pathogen relationship. Plasmids are a recognized key driver of rapid evolution, providing the means to acquire genes encoding novel metabolic pathways, or gain resistance toward metals and antibiotics via horizontal gene transfer (Schierstaedt et al., 2019). Similarly, genes related to pathogenicity and host defense can be acquired through plasmid encoded genes. However, to fully understand the complex network of communication between host plants, endophytes and pathogens/predators, it is necessary to take on a holistic approach that encompasses many different-Omic disciplines rather than pursuing a reductionist view and investigating individual components in isolation (Kaul et al., 2016; Pinski et al., 2019). Genome sequencing and subsequent comparative genome analyses will provide the necessary foundation to develop novel hypotheses to be validated by a wide spectrum of -omics technologies. While research on the genetic blueprint is slowly emerging, it often is limited to the investigation of microbiome phylogeny via 16S rRNA and housekeeping gene sequencing (Kumar et al., 2018; Liotti et al., 2018; Kang et al., 2019) or high-level biochemical characterization *via* proteome and metabolome studies (López et al., 2018). Genomic analysis of *Bacillus* spp. strains has been proposed as being critical to understanding the complex and intricate plant host-microbiome-(insect) pathogen communication network (Lopes et al., 2018). Transcriptomics datasets enable the study of changes in gene expression over

time, as endophytes and their hosts encounter environmental changes such as insect pests. However, the varying levels of gene expression do not reveal the function of the resulting proteins and enzymes and proteomic/metabolomic approaches are required to relate the genomic diversity to the functionality of microbes (Rasmussen et al., 2012; Afroz et al., 2013). Studies into the *M. sativa* interactome first emerged a decade ago (Rodríguez-Llorente et al., 2009). These described the symbiosis interactome between the model bacterium *Sinorhizobium meliloti* with its legume host using computational methods and models that provided a theoretical basis, but lacked experimental validation.

## PRODUCTION AND PERSISTENCE

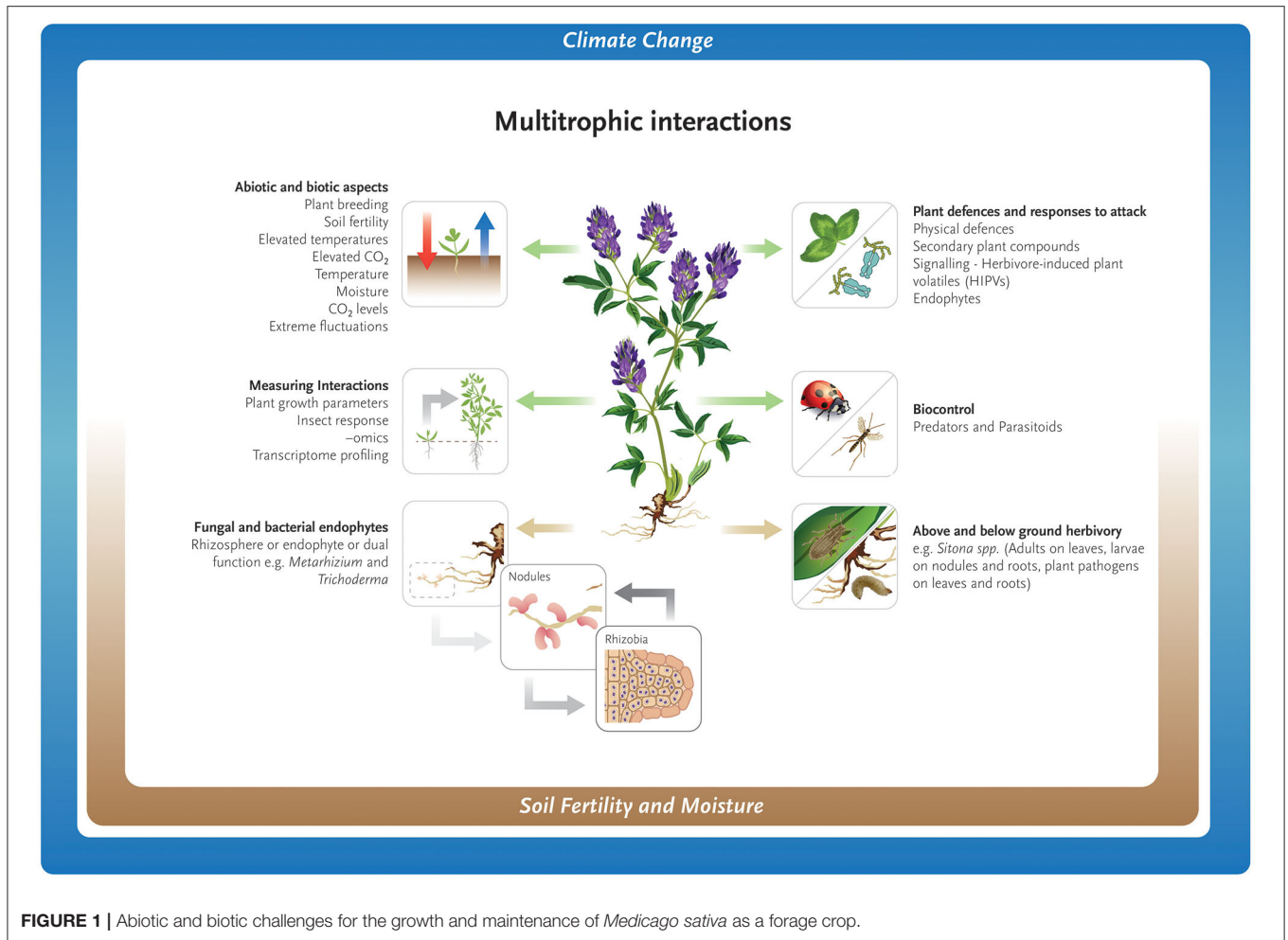
As a forage crop with a worldwide distribution, breeding of germplasm to adapt to a range of climates has been essential to achieve sustainable high quality forage production for livestock (Undersander et al., 2011). Conventional plant breeding techniques (e.g., selective breeding, cross breeding, male sterile line breeding), have targeted yield, quality, persistence under repeated grazing or cutting, heat and cold tolerance, salinity and salt tolerance, drought, high moisture, and insect pest and disease resistance (Bouton, 2012; Scasta et al., 2012; Annicchiarico et al., 2015; Shi et al., 2017). Selection of salinity-tolerant actinobacteria has been demonstrated to improve rhizobia function and overall *M. sativa* production under salt stress (Saidi et al., 2021). As the demand for meat-based proteins increases in response to human population growth, so too has the environmental limits under which *M. sativa* is being grown. This has led to evaluation of genotypes adapted for subtropical conditions (Acharya et al., 2020; Hoppen et al., 2022), for both high altitude and latitude (Shi et al., 2017), and environments with high temperature and low soil moisture (del Pozo et al., 2017; Lemaire et al., 2019).

Environment plays a critical role to determining yield and persistence (Li et al., 2010). Pembleton et al. (2010) showed that under a cool temperate climate, environment had a significant impact on the persistence and yield of alfalfa genotypes, with winter active genotypes having low persistence and DM yield. Breeding for grazing tolerance has been shown to be important in persistence (Sewell et al., 2011; Harvey et al., 2014; Burnett et al., 2020), but there can be a trade off in production, with winter active germplasm generally showing poor persistence (Humphries et al., 2006; Harvey et al., 2014). What is apparent, is that as the worldwide range of *M. sativa* expands, breeding programs to meet regional conditions will become more important (Bouton, 2012; Shi et al., 2017).

## FUTURE RESEARCH

This review has examined several aspects of plant–insect–microbe–interaction, and highlighted their complexity. In relation to *M. sativa*, there are research opportunities to address important gaps in our understanding of the biotic and abiotic parameters that can be exploited to improve the significance and utility of the plant. This includes the role of endophytes on growth, colonization and transmission,

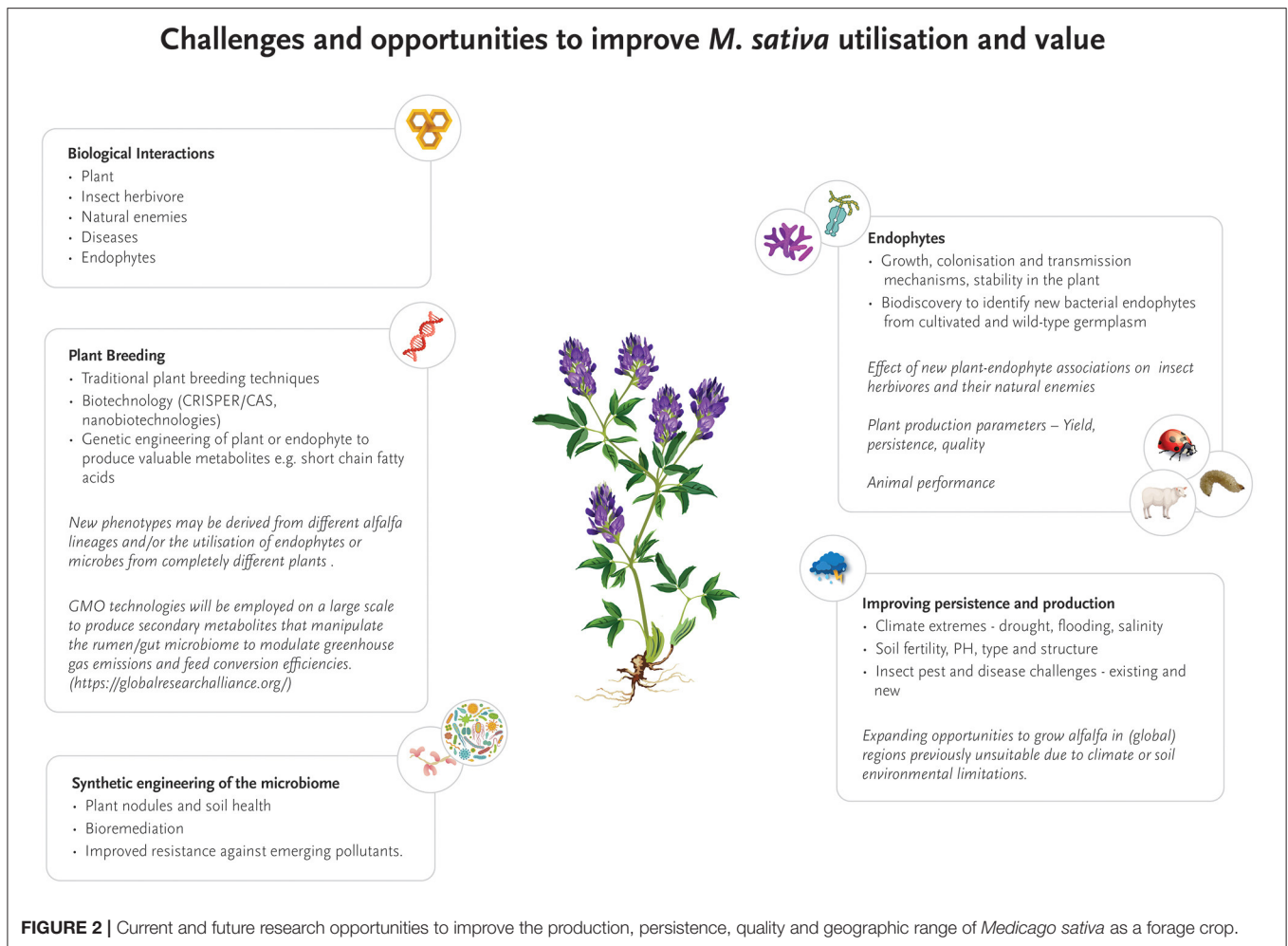




subsequent persistence, their prevalence in *M. sativa* and their impact on insect herbivory and pathogens. Bioprospecting for endophyte strains that mediate resistance outside cultivated strains would also seem worthwhile. *Medicago sativa* is believed to have originated in the Caucasus region: north-eastern Turkey, Turkmenistan and north-western Iran (Michaud et al., 1988). Surveys in these regions may yield valuable “wild-type” germplasm containing bacterial endophytes or traits that provides tolerance or resistance to certain pests and diseases. China also has potential unique *M. sativa* germplasm (Shi et al., 2017), that may provide ecotype–endophyte associations with protection from insect pests and diseases. However, while this approach would intuitively seem a useful strategy, it may not necessarily yield the desired outcome. For instance, *M. sativa* and alfalfa weevil (*H. postica*) are sympatric in Turkey, but a search for plants naturally resistant to the weevil were unsuccessful (Ratcliffe and Campbell, 1995), although resistant germplasm has been reported in Iran (Abbasi, 2020). Other factors also need consideration, including the abiotic environment and its influence on plant responses to pests and pathogens. Increasing agricultural intensification has been found to have a strong negative association with root fungal communities (Banerjee

et al., 2019). Fertilizer application may have a negative effect on bacterial endophytes associated with *M. sativa*, as has been demonstrated in grasses (Wemheuer et al., 2016). Conversely, rhizobia need critical mineral nutrients for metabolic processes to enable their survival and growth as free-living soil saprophytes, and in their symbiotic relationship with legumes (O’Hara, 2001). Non-rhizobia found in the rhizosphere and nodule microbiome are also critical to nodule formation and legume fitness (Schaedel et al., 2021). The impact of grazing or cutting on the ability of *M. sativa* to respond to pest and pathogen attack are also areas for potential research.

Climate change presents an obvious challenge, not only because there will be more instability in climatic events such as drought or floods, but economically valuable plant species will potentially be exposed to new pests and plant diseases due to range expansion, (Trebecki et al., 2017; Ricciardi et al., 2021), either through natural dispersal or spread along trade and tourism pathways (McNeill et al., 2021). Elevated temperatures will have both negative and positive consequences for biological control (Thomson et al., 2010; Gerard et al., 2013), and plant defense responses to pathogens (Venkatesh and Kang, 2019). Creating crop varieties that are highly attractive to natural



pest enemies has been suggested as a simple way to achieve better biological control of pests (Dhandaydham et al., 2008; Dicke and Baldwin, 2010). Beyond traditional plant breeding approaches (Annicchiarico et al., 2015; Capstaff and Miller, 2018), gene editing technology has been proposed as a way of improving the adaptability of plants to challenging abiotic and biotic conditions (Sanzari et al., 2019; Demirer et al., 2021). Transgenic plants with desirable traits for growth or pest resistance are already available (e.g., cotton or maize) and applicable to *M. sativa*. For example, under laboratory conditions Wang et al. (2019) demonstrated that by insertion of a gene expressing an aphid alarm pheromone into *M. sativa*, repellency by *A. pisum* to transformed plants was shown in subsequent behavioral tests. Technologies such as CRISPR/Cas genome editing (Cobb et al., 2019; Pixley et al., 2019), nanotechnologies (Sanzari et al., 2019; Behl et al., 2022), or the combination of both nanotechnologies with CRISPR-Cas technology (Demirer et al., 2021), has also been suggested as a means of improving plant production, quality, and persistence under challenging environments. Nanotechnology may also provide a means of monitoring crops by translating plant chemical signals into digital information that can be monitored by standoff electronic

devices (Giraldo et al., 2019). While mainly conceptual, synthetic engineering of the phytomicrobiome has also been suggested as a way of improving plant performance (Ke et al., 2021). These new technologies will also require both regulatory and social license to operate, as well as biological-based research to understand relationships and benefits across trophic levels and different environments (Sanzari et al., 2019; Behl et al., 2022). Understandably, adoption of these technologies will be variable on a global scale as dictated by government and public timeframes.

Although some progress has been made to increase insect resistance to crops, research on *M. sativa* still provides opportunities for improvement in this important field. Most notably, the impact of bacterial endophytes on plant resistance to pests and pathogens is yet to be fully explored, despite the attractiveness of this target with regards to rapid evolution and genetic engineering (Figure 2). This knowledge gap opens significant opportunities in the future for new research that focuses on unraveling the genetic and metabolic networks between bacterial endophytes, host plants and insect pest and beneficial species, with the aim to engineer or naturally evolve bacterial endophytes with enhanced insecticidal properties. As



a first step and to build the fundamentally required genomic knowledge base, a comprehensive catalog of genomes of known *M. sativa* bacterial endophytes needs to be established, preferably from microbiomes isolated from a range of agricultural *M. sativa* growth regions worldwide and wild-type relatives. Based on this foundation, transcriptomic and metatranscriptomic analyses will unravel how bacterial gene expression changes over time and to plant and insect exposure. In a second step, changes in gene expression levels of the host plant and insect predators can be quantified and correlated to bacterial endophytes. Novel insights gained from these experiments will be critical in understanding key genetic building blocks of insect resistance. Metaproteomic and metabolomics studies will translate the genetic basis into identifying the presence, absence and modification of biologically active compounds, and their concomitant effects on both plant and associated insect pests (Zogli et al., 2020). Once the intricate network of interactions and metabolic responses is better understood, bacterial endophyte strains may be directly evolved toward specific metabolic changes that will specifically enhance insect resistance—or target other limitations of *M. sativa* cultivation such as drought or salinity.

## CONCLUSION

A broader, inter-disciplinary approach that simultaneously addresses plant-insect-endophyte-plant pathogen interaction and their impacts viewed against abiotic and biotic aspects is desirable, as it concurrently addresses a range of research questions (Raffa et al., 2020). The benefits are research advances and outcomes that support agricultural resilience, industry stakeholders and farmers. However, achieving this aim obviously depends on appropriate research to allow *M. sativa* to achieve its potential in a challenging environment.

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## AUTHOR CONTRIBUTIONS

MM, XT, EA, WB, and SS contributed to the development of the concepts discussed in this review. All authors contributed to writing the manuscript. All authors contributed to the article and approved the submitted version.

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