Agriculture, Ecosystems and Environment 233 (2016) 55–59

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

Short communication

Effect of rhizobia symbiosis on lignin levels and forage quality in alfalfa (*Medicago sativa* L.)

Zhiqiang Zhang[a,b], Linhui Shao[c], Leqin Chang[a], Yuman Cao[a], Tong Zhang[a], Yafang Wang[a], Yushi Liu[a], Pan Zhang[a,d], Xiaoqin Sun[e], Yajun Wu[f], Tianming Hu[a,*], Peizhi Yang[a,*]

[a] Department of Grassland Science, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, Shaanxi, China
[b] College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Huhhot 010000, Inner Mongolia, China
[c] Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China
[d] Department of Grassland Science, College of Animal Science and Technology, Northeast Agricultural University, Harbin 150030, China
[e] Department of Animal Science, College of Animal Science and Technology, Northwest A&F University, Yangling 712100, Shaanxi, China
[f] Department of Biology and Microbiology, South Dakota State University, Brookings, SD 57007, USA

ARTICLE INFO

Article history:
Received 27 April 2016
Received in revised form 30 August 2016
Accepted 31 August 2016
Available online xxx

Keywords:
Alfalfa
Lignin
Medicago sativa
Rhizobia symbiosis
Forage quality

ABSTRACT

Many beneficial effects of symbiotic rhizobia on leguminous plants have been reported. Here we report a novel effect of rhizobia on forage quality in alfalfa. We found that nodulated alfalfa showed an increase in lignin content and a decrease in digestibility in comparison with non-nodulated plants. Detailed studies revealed that nodulation resulted in an increase in monolignol G unit and S unit. An overall increase in lignin content in nodulated alfalfa was associated with more lignified tissues in the stem and an upregulation of transcript levels of several lignin biosynthesis genes. We hypothesize that an increase in lignin content in nodulated alfalfa is a result of defensive response in plants to rhizobial invasion.

Alfalfa (*Medicago sativa* L.), one of the most important leguminous forage crops worldwide, can grow in nitrogen-poor soils through symbiotic nitrogen fixation by rhizobia in root nodules. In addition to nitrogen fixation, rhizobia have been shown to benefit legume plants in many other ways. Rhizobia can stimulate seed germination, influence plant development, promote plant growth, improve grain yields, as well as increase photosynthetic rates (Dakora, 2003). In addition, rhizobia are known to suppress the population of soil pathogens (Berendsen et al., 2012). Recent studies showed that plant-associated rhizobia can enhance some degree of tolerance in plants to abiotic stresses (Grover et al., 2011; Shrivastava and Kumar 2015).

The legume-rhizobia association is initialized by flavonoid and isoflavonoid compounds secreted by legumes (Long, 2001), flavonoid secretion can be impacted by lignin modification (Gallego-Giraldo et al., 2011). Several reports have shown that soil microbiota such as bacteria and fungi could change lignin deposition in plants (Parrott et al., 2002; Bennett et al., 2015). Recent studies showed that the lignin content in both shoots and roots of barley could be altered by soil microbial community (Bennett et al., 2015). This leads to our question whether symbiotic rhizobia would impact lignin content in forage legume plants. Lignin content is negatively correlated with digestibility due to its adverse influence on forage digestibility in ruminant animals (Jung et al., 1997). We hypothesize that rhizobium symbiosis will increase the lignin content and decrease forage quality in alfalfa.

In this study, alfalfa (*M. sativa* L. cv. baoding) plants were inoculated with *Rhizobium meliloti* strain Dormal (NA) or were not inoculated (NN), as described previously (Yang et al., 2013). Inoculated plants were irrigated with a nitrogen free nutrient solution, while NN plants were irrigated with a full nutrient solution. After 8 weeks, many pink nodules were observed in the roots of NA plants, and no nodules were present in the roots of NN plants (Fig. 1). Lignin content in alfalfa shoots was measured by the acetyl bromide procedure on 8-week old plants (Chang et al., 2008). Inoculated plants showed a 47% increase in lignin content

Abbreviations: PAL, phenylalanine ammonialyase; C4H, cinnamate 4-hydroxylase; C3H, coumaroylshikimate 3′-hydroxylase; HCT, hydroxycinnamoyl-CoA: shikimate/quinatehydroxycinnamoyltransferase; CCR, cinnamoyl-CoA reductase; IVTD, in vitro true digestibility; NDF, neutral detergent fiber; ADF, acid detergent fiber.

* Corresponding authors.
E-mail addresses: hutianming@126.com (T. Hu), yangpeizhi@126.com (P. Yang).

http://dx.doi.org/10.1016/j.agee.2016.08.035
0167-8809/© 2016 Published by Elsevier B.V.

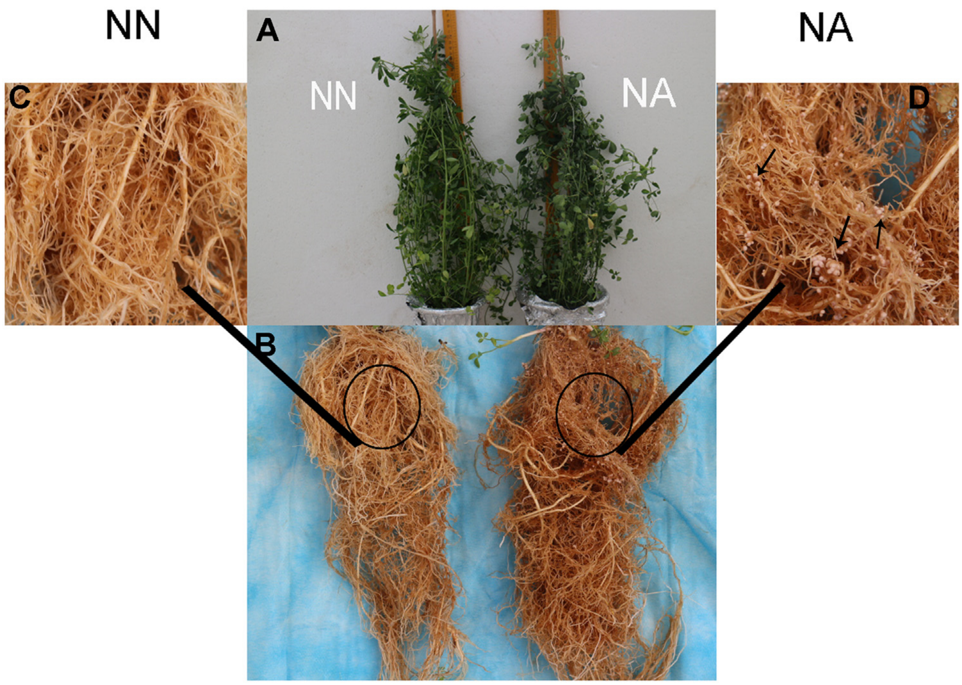

Fig. 1. Overall plant growth and roots from non-nodulated (NN) and nodulated (NA) alfalfa after growth for 8 weeks. (A) Aboveground tissues of NN and NA plants; (B) roots of NN and NA plants; (C, D) Closeup of roots of NN (C) and NA (D) plants. Black arrows show the pink nodules in the roots of NA plants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared with NN plants (Fig. 2B). Stem cross-sections of the fourth internodes in alfalfa were treated with Wiesner reagent (phloroglucinol-HCl) which specifically stains lignin (Guo et al., 2001). Inoculated plants exhibited increased staining of the xylem areas in the internodes compared to NN plants (Fig. 2C). In addition, clearer and stronger lignin staining was observed in the phloem fiber of NA stem, but little in NN stem (Fig. 2C).

Lignins can be classified based on the content of each of the three lignin monomers, hydroxyguaiacyl (H), guaiacyl(G) and syringyl (S) (Boerjan et al., 2003). To investigate how lignin composition was impacted by rhizobium symbiosis, the amount of each lignin monomer was determined by using the thioacidolysis method (Guo et al., 2001). Inoculated plants exhibited an increase in G unit (21.3%) ($P < 0.01$) and S unit (11.4%) ($P < 0.05$) but no significant change in H units compared to NN, resulting in a decrease in S/G ratio from 1.23 in NN plant to 1.03 ($P < 0.05$) in NA plants (Fig. 2A).

Lignin decreases forage quality used for ruminants feed (Jung and Deetz 1993). Therefore, we examined whether the greater lignin content in NA will lead to a change in alfalfa quality using the *in vitro* true digestibility (IVTD) of dry matter assay (Getachew et al., 2001; Reddy et al., 2005). Inoculated plants displayed a nearly 26% reduction in digestibility (IVTD) compared with NN ($P < 0.05$) (Fig. 3B), which may be due to the increase of lignin content and the S/G ratio (Fig. 2A). We also quantified crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL), which are important indices for forage quality and were also quantified (Sanz-Sáez et al., 2012). Crude protein content was estimated as N% $\times$ 6.25 (Chang et al., 2008), while NDF, ADF and ADL were determined using the ANKOM Filter Bag System described by Vogel et al. (1999) and Reddy et al. (2005). Inoculated plants showed higher NDF, ADF, and hemicellulose levels compared with NN (Fig. 3A,B). Lignin accumulation in NA might have resulted in the increase of NDF and ADF (Sanz-Sáez et al., 2012). Although a strong, negative, linear relationship existed between forage digestibility and ADL level (Reddy et al., 2005), ADL level was not different between NA and NN in this study. These results suggest that a reduction in IVTD in NA may be mainly caused by other kind of lignins or fibers. However, the overall biomass production, other phenotypic characteristics (Table 1), and CP content (Fig. 3A) were not different between NA and NN plants. Forage quality was likely changed due to the alteration of lignin and other cell wall components induced by rhizobia symbiosis.

To gain some understandings of the molecular basis of lignin accumulation in NA plants, we examined expressions of several lignin biosynthesis genes in response to rhizobia symbiosis in alfalfa using a quantitative real-time PCR method as described previously (Zhang et al., 2015). The primer sequences for transcript analyses are shown in Table 2. Among the eight genes studied, the transcript levels of *PLA* (phenylalanine ammonialyase), *C4H* (cinnamate 4-hydroxylase), *C3H* (coumaroyl shikimate 3′-hydroxylase), *HCT* (hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyl transferase), and *CCR* (cinnamoyl-CoA reductase) genes were upregulated in NA compared to NN ($P < 0.05$), while the transcript levels of *4CL* (4-coumarate: CoA ligase), *COMT* (caffeic acid O-methyltransferase), and *CAD* (cinnamyl alcohol dehydrogenase) genes were not affected (Fig. 2D). These results suggested that the higher transcript levels *of PAL*, *C4H*, *C3H*, *HCT* and *CCR* genes may have contributed to the increase in lignin content in NA plants.

Plant mutualists, such as rhizobia and mycorrhizal fungi, were thought to impact lignin biosynthesis through influencing the delivery of plant nutrients (Moura et al., 2010). Therefore, levels of nutrient elements such as carbon (C), nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) were determined in NN and NA plants as described by David (1960) and Fu et al. (2014). No significant difference was observed in the C, N and P levels between NA and NN (Fig. 3C), suggesting that the changes in lignin levels were not induced by C, N and P levels. However, the level of K in NA was significantly lower than that of NN, while the level of Ca was significantly higher in NA compared to NN ($P < 0.05$) (Fig. 3C). Increased Ca levels have been shown to stimulate the activity of guaiacol-POD and PAL in wheat (Kolupaev et al., 2005), resulting in