Trends in Plant Science

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Opinion

A Model for Nitrogen Fixation in Cereal Crops

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Nitrogen-fixing microbial associations with cereals have been of intense interest for more than a century (Roesch *et al.*, *Plant Soil* 2008;302:91–104; Triplett, *Plant Soil* 1996;186:29–38; Mus *et al.*, *Appl. Environ. Microbiol.* 2016;82:3698–3710; Beatty and Good, *Science* 2011;333:416–417). A recent report demonstrated that an indigenous Sierra Mixe maize landrace, characterized by an extensive development of aerial roots that secrete large amounts of mucilage, can acquire 28–82% of its nitrogen from atmospheric dinitrogen (Van Deynze *et al.*, *PLoS Biol.* 2018;16: e2006352). Although the Sierra Mixe maize landrace is unique in the large quantity of mucilage produced, other cereal crops secrete mucilage from underground and aerial roots and we hypothesize that this may represent a general mechanism for cereals to support associations with microbial diazotrophs. We propose a model for the association of nitrogen-fixing microbes with maize mucilage and identify the four main functionalities for such a productive diazotrophic association.

Highlights

A novel mechanism for nitrogen fixation in an indigenous maize landrace was recently reported that focused on a nitrogen-fixing microbiota supported by an abundant secreted mucilage.

The structure of a complex polysaccharide comprising the mucilage suggested that terminal fucose, arabinose, and xylose may provide the energy source to fuel nitrogenase activity.

Role for Nitrogen Fixation in Cereal Crops

Nitrogen-fixing microbial associations with nonlegumes, especially cereals, have been a topic of intense interest for more than a century, since such associations could reduce the requirement for nitrogen fertilizers [1–4]. For instance, sugarcane associates with diazotrophic endophytes that contribute to its nitrogen nutrition in some environments [5–7] and a study based on ^{15}N dilution experiments in *Miscanthus* × *giganteus* concluded that this bioenergy feedstock could acquire about 16% of its nitrogen from the air [8]. These examples indicate that some monocots have the potential to associate with diazotrophs and acquire small, but significant, amounts of fixed nitrogen from the atmosphere. It has been shown that the model C_4 grass *Setaria viridis* can obtain most of its fixed N from associative nitrogen fixation following inoculation with diazotrophs [9]. The possibility that cereal crops obtain a significant proportion of total N by associative nitrogen fixation has also been suggested by a 50-year assessment of global nitrogen budgets in maize (*Zea mays*), rice (*Oryza sastiva*), and wheat (*Triticum aestivum*), which concluded that up to 24% of the total nitrogen in these crops was derived from nonlegume symbiotic nitrogen fixation [10]. Interestingly, mucilage on aerial roots of sorghum (*Sorghum bicolor*) with an inset micrograph of *Azospirillum brasilense* was highlighted on the cover page of the proceedings of a conference on cereal nitrogen fixation held in India in 1984 [11], suggesting that it was suspected more than 35 years ago that sorghum mucilage harbors a diazotrophic microbiota. However, no report on nitrogen fixation in sorghum mucilage was published in the 1984 proceedings volume or elsewhere.

A recent report demonstrated that an indigenous landrace of maize found in Totontepec Villa de Morelos in the Sierra Mixe region of Mexico acquires 28–82% of its nitrogen from the air. This conclusion was supported by multiple techniques to evaluate nitrogen fixation: acetylene reduction assays (ARAs), ^{15}N natural abundance, ^{15}N dilution, ^{15}N gas enrichment, and nitrogen balance experiments [12]. Interestingly, unlike most modern maize varieties, this Sierra Mixe maize develops extensive aerial roots, which secrete large amounts of mucilage after rain (Figure 1). This mucilage is rich in arabinose, fucose, and galactose and harbors a diazotrophic microbial community, which was concluded to be at least partially responsible for the fixation and delivery of atmospheric nitrogen to the maize plant [12]. This conclusion suggests that mucilage is an

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Figure 1. Aerial Root Mucilage. The aerial roots of Sierra Mixe maize secrete large quantities of a viscous mucilage between 3 and 6 months after planting. Photograph credit: Valentina Infante, with permission.

essential determinant of nitrogen fixation in the Sierra Mixe maize landrace and suggests that mucilage secretion may play a more general role in harboring diazotrophic microbial communities in other cereals as well. The role of mucilage in providing an environment for nitrogen fixation has also been proposed in various biological systems, including the *Gunnera–Nostoc* and the *Azolla–Anabaena* symbioses [13,14]. Here, we propose a general model for the functional

plant–microbe association supporting diazotrophic activity and suggest that this may be a general feature supporting nitrogen fixation in cereal crops. This model is intended to provide a framework to evaluate diazotrophic activity in cereal crops and, potentially, to increase nitrogen fixation in cereals by optimizing these mucilage functionalities through genetic selection and/or the structure of mucilage-associated microbial communities.

Occurrence and Functions of Mucilage

A key feature of the Sierra Mixe maize landrace mucilage is the abundance of sugars that potentially serve as a source of energy for the diazotrophs. The monosaccharide composition indicated that the mucilage primarily comprises fucose (41%), galactose (36%), arabinose (14%), xylose (3%), glucuronic acid (3%), and mannose (3%) [12]. This composition is similar to the mucilage reported for maize underground roots [15,16]. Osborne *et al.* found that underground root mucilage contained fucose (61.0%) and glucose (31.4%), while Chaboud found the mucilage to contain galactose (30.7%), fucose (19.3%), glucose (18.5%), xylose (15.2%), and arabinose (13.4%). Both of these previous studies showed high amounts of fucose, xylose, arabinose, and galactose, which agrees qualitatively with the analysis of the Sierra Mixe aerial root mucilage. This monosaccharide composition is not commonly found in plant cell wall polysaccharides and may play a role in signaling associative diazotrophic bacteria that can degrade the mucilage complex polysaccharide and use the released monosaccharides to support growth and nitrogen fixation.

The majority of Sierra Mixe mucilage sugars are ethanol insoluble, indicating that they comprise a large polysaccharide. In a recent report, the ethanol-insoluble polysaccharide structure was characterized using a variety of novel LC-MS-based methods to arrive at a proposed structure [17] (Figure 2). The mucilage polysaccharide comprises a 2-linked galactose backbone with radiating fucose and xylose residues as well as long chains of 2-linked arabinose. While it is possible that this 'parent polysaccharide' is a mixture of different polysaccharides, this was ruled out by anion exchange chromatography, which indicated that the mucilage comprises a single major polysaccharide [17]. Of particular interest are the abundant terminal fucose, xylose, and arabinose residues, whose enzymatic release could serve to 'feed' the mucilage-resident microbiota, including diazotrophs. This elucidation of the mucilage polysaccharide structure has provided a valuable insight into understanding the mechanisms of the diazotrophic association underlying the Sierra Mixe maize apparent nitrogen fixation.

Exudates are a common feature of all plant roots [18] and comprise a wide range of both low-molecular-weight (amino acids, organic acids, sugars, and phenolic compounds) and high-molecular-weight (polysaccharides and proteins) compounds [19]. These exudates have a range of functions including disease suppression, increased nutrient uptake, increased tolerance to abiotic stress, and structuring of the plant–soil microbiome [18–21]. In *Azospirillum* species, for example, chemotaxis is the first stage directing the bacteria toward the root exudates, followed by attachment. Bacterial capsular polysaccharides initially mediate the attachment; previous studies identified a lectin present in the capsular polysaccharide of *A. brasilense* Sp7 with specificity for L-fucose and D-galactose [22]. Further studies characterized a sugar-binding protein involved in the uptake of D-galactose, functioning in the chemotaxis of *Azospirillum* toward D-galactose, L-arabinose, and D-fucose [23]. Lectins are sugar-binding proteins that specifically and reversibly recognize and bind to carbohydrates on the root surface. Following attachment, an aggregation step is mediated by bacterial lipopolysaccharides (LPSs), in which L-arabinose was demonstrated to be the dominant sugar in the *A. brasilense* LPS composition [24]. The mucilage polysaccharides are likely to replicate the optimal environment to sustain specific microbiota, not only by providing an energy source but also by supporting bacterial metabolism and essential colonization mechanisms for their establishment in the mucilage–aerial root environment.

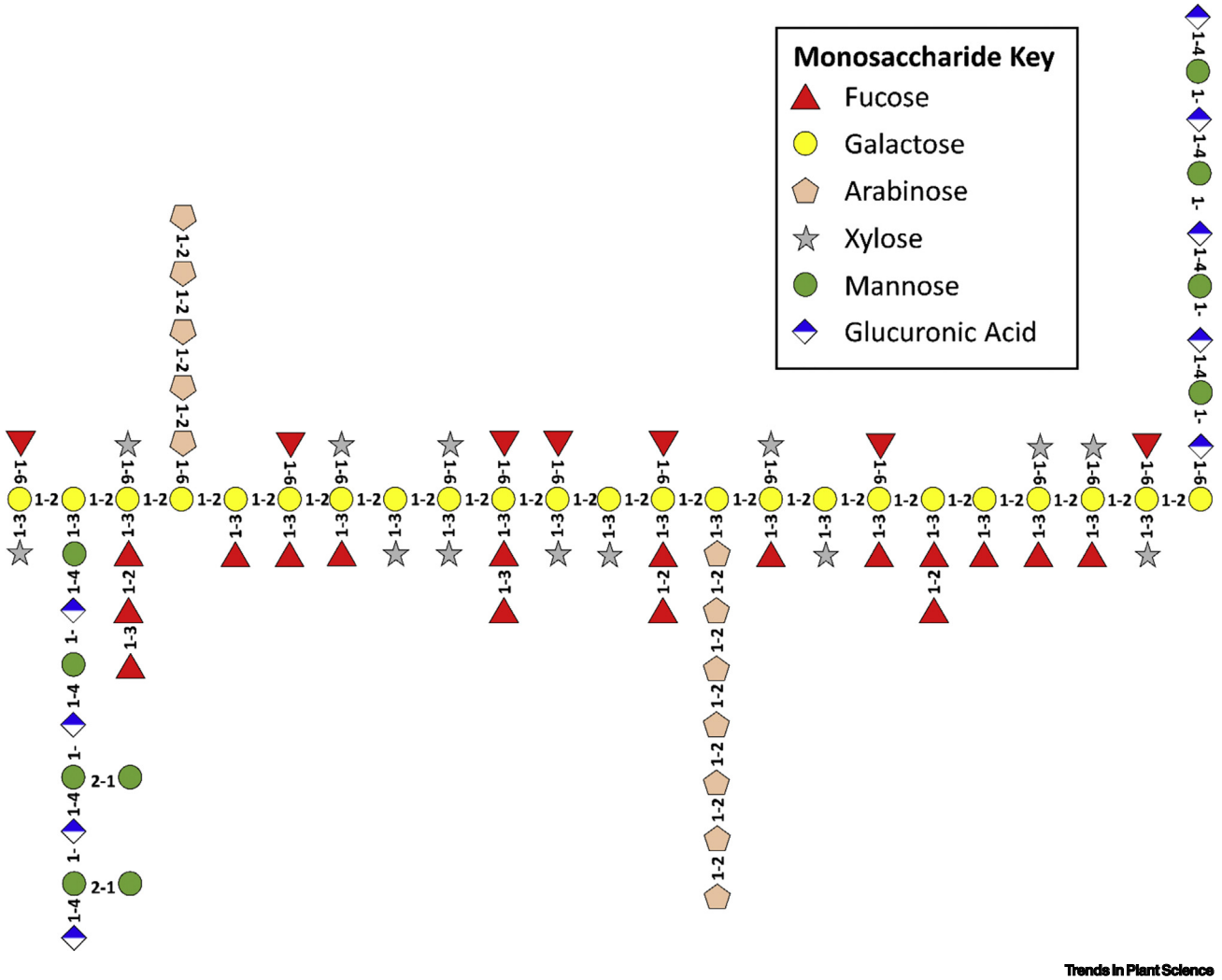

Figure 2. The Proposed Mucilage Polysaccharide Structure. Polysaccharide comprises a 2-linked galactose backbone containing xylose and fucose decoration along with arabinan and mannogalacturonan side chains. Reprinted, with permission, from [17]. Requests for further permission to use this material should be directed to the ACS.

The low-molecular-weight compounds account for much of the diversity of root exudates while the high-molecular-weight components such as the mucilage polysaccharides comprise the larger proportion by mass. As one measure of the importance of root exudates, it has been estimated that roots release 5–21% of the plant's photosynthate as soluble sugars, amino acids, or secondary metabolites, which are used to recruit or support microbial communities in the rhizosphere [18,19,25,26]. In addition to the mucilage exudate characterized in Sierra Mixe aerial roots, mucilage has also been described in maize underground roots [27] as well as in other cereal crops including wheat, barley, and sorghum [28–30] suggesting that mucilage is likely to be a common feature of cereal crop roots. Although there is no structural information on the mucilage polysaccharide other than in Sierra Mixe maize, the wheat root mucilage was visualized using an antifucose antibody [28], which would be consistent with the terminal fucose residues found in the maize polysaccharide structure. Some sorghum accessions also produce large numbers of aerial roots similar to the Sierra Mixe maize, and sorghum aerial roots can produce copious amounts of mucilage [11,31].

A Model of a Mucilage-Supported Diazotrophic Microbiota

Based on what has been reported, we can propose a model of the mucilage-supported diazotrophic microbiota in terms of the major functions that must be fulfilled by the microbial community and plant partner. The general requirements for the support of a nitrogen-fixing community of microbes are an abundant supply of sugars or organic acids, a low-oxygen environment, and a low-nitrogen environment. Based on the structural knowledge of the mucilage polysaccharide, we can predict that the plant together with the diazotrophic microbiota should fulfill the following four primary functions: (i) disassembly of the complex polysaccharide to release terminal fucose, arabinose, and xylose residues, by bacteria and plant enzymes; (ii) utilization of the released fucose, arabinose, and/or xylose monosaccharides to fuel nitrogenase activity; (iii) reduction of oxygen tension; and (iv) lowering of nitrogen levels by plant uptake from the mucilage (Figure 3).

Disassembly of the Complex Mucilage Polysaccharide

The structure of the mucilage polysaccharide highlights the particular abundance and terminal positions of fucose, arabinose, and xylose residues suggesting that their enzymatic release

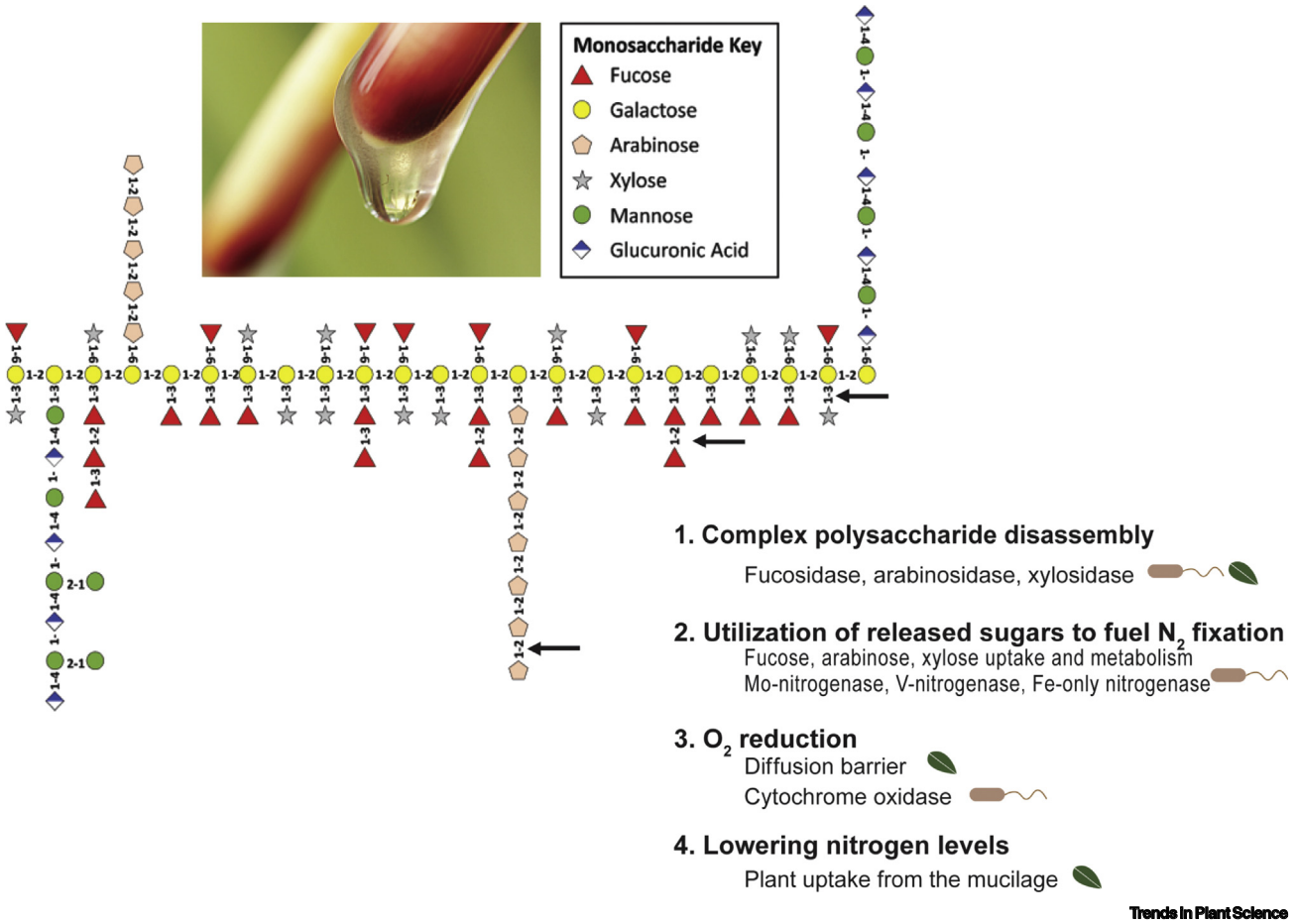

Figure 3. Proposed Model of the Major Functionalities of the Mucilage-Supported Diazotrophic Microbiota of the Sierra Mixe Aerial Root Mucilage. Green symbols indicate functionality contributed by the plant and brown bacterium-shaped symbols indicate functionality contributed by the microbiota. Arrows indicate cleavage sites to release terminal monosaccharide residues. Carbohydrate structural model reprinted, with permission, from [17]. Requests for further permission to use this material should be directed to the ACS.

could serve to feed the mucilage-resident microbiota including diazotrophs. The presence of endogenous glycosyl hydrolases in Sierra Mixe mucilage was assessed utilizing 4-nitrophenyl (4NP)-conjugated glycosides (4NP-β-Glc, 4NP-β-Xyl, 4NP-β-Gal, 4NP-β-Man, 4NP-α-Fuc, 4NP-α-Xyl, 4NP-α-Man, 4NP-α-Ara) and was reported previously [32]. The highest enzymatic activity observed in the mucilage was determined to be from enzymes acting on the 4NP-α-arabinofuranoside substrate followed by 4NP-β-xylopyranoside, 4NP-α-mannopyranoside, and 4NP-β-galactopyranoside substrates, each supporting similar levels of 4NP liberation. Endogenous enzymes that facilitate the release of 4NP residues from 4NP-α-fucopyranoside substrates showed the fifth-highest glycosidase activity rate in these assays (Figure 4). This report revealed the presence of active enzymes likely to be involved in the disassembly of the mucilage parent polysaccharide and the potential release of terminal arabinose, xylose, and fucose residues. Further studies will be required to determine whether the endogenous glycosyl hydrolase activities detected in the mucilage are due to microbial (bacterial or fungal) and/or plant enzymes. Irrespective of the source of glycosyl hydrolases, we propose that this is an essential activity to support the mucilage-associated diazotrophic microbiota.

Sugar Utilization to Support Nitrogenase Activity

The proposed mucilage-resident diazotrophic microbiota must comprise, in part, microbes with active nitrogenase activity and the capacity to utilize the sugars that are abundantly represented in the complex mucilage polysaccharide, fucose, arabinose, and xylose.

It was previously shown that the Sierra Mixe mucilage contains microbial genes encoding the minimum gene set (*nif* genes) for nitrogen fixation by molybdenum nitrogenase [12,33]. This molybdenum nitrogenase is likely to be a critical component for nitrogen fixation in the Sierra Mixe mucilage, but two alternative nitrogenases, vanadium nitrogenase and iron-only nitrogenase, have been identified that may contribute to nitrogen fixation in the mucilage [3]. Only three bacteria have been shown to have genes and activity for all three nitrogenases [34], but these combinations of nitrogenases may operate under a broader range of conditions or with higher

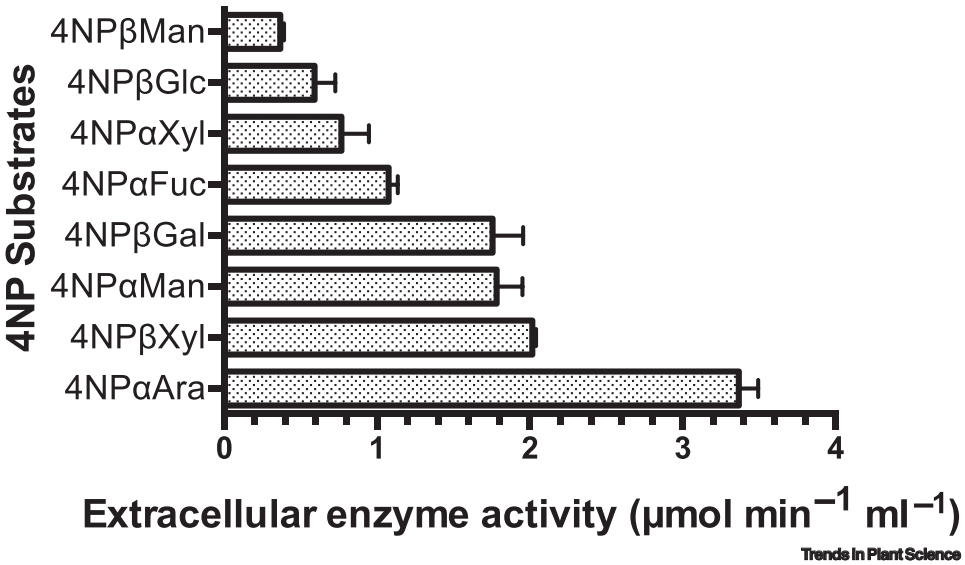

Figure 4. Detection and Quantification of Glycosyl Hydrolase Activity Endogenous to Aerial Root Mucilage. Freshly collected mucilage was spiked with different 4-nitrophenyl glycoside substrates to assess the hydrolytic activity of endogenous mucilage enzymes. Reprinted from [32].

overall activity. We have identified bacterial isolates from mucilage, which similarly encode all three nitrogenases (unpublished). It will be interesting to determine to what extent these diazotrophs have access to molybdenum, vanadium, or even iron in the mucilage.

In addition to the capacity to fix atmospheric nitrogen, this model predicts that the diazotrophs can fuel this fixation by utilizing sugars that are abundantly represented in the mucilage polysaccharide; namely, fucose, arabinose, and/or xylose. The diazotroph *Herbaspirillum seropedicae*, for example, cannot naturally metabolize disaccharides [35] and preferably uses monosaccharides such as xylose to synthesize its storage carbon polymer polyhydroxybutyrate (PHB) [36]. It was recently demonstrated that PHB metabolism supports specific metabolic routes to support plant growth promotion by *H. seropedicae* [37]. Interestingly, PHB mobilization in rhizobia bacteroids provides energy and reducing power for nitrogen fixation [38]. In *Escherichia coli*, glucose and lactose are preferred substrates, but in their absence and the presence of xylose and arabinose, *E. coli* will consume arabinose preferentially until it is depleted and then will also consume xylose [39]. The human gut also provides some analogy to mucilage in that fucose is abundant as a component of human milk oligosaccharides, mucins, and other glycoconjugates. The genome of a probiotic bacterium, *Lactobacillus rhamnosus*, carries genes encoding a putative L-fucose permease (*fucP*) as well as encoding the L-fucose catabolic pathway [40]. Similarly, *Bifidobacterium longum* subsp. *infantis* preferentially consumes human milk oligosaccharides that are abundant in early lactation and this bacterium encodes one large locus that contains all of the glycosidases (sialidase, fucosidase, galactosidase, and hexosaminidase) and transporters necessary for disassembling, importing, and metabolizing human milk oligosaccharide components [41]. We hypothesize that the mucilage diazotrophs have and express genes needed for the import and metabolism of mucilage polysaccharide components to produce ATP to fuel the energy-expensive nitrogenase activity.

Reduction of Oxygen Levels in the Mucilage Environment

Nitrogen fixation in aerobic bacteria is a balancing exercise in the sense that it requires high levels of ATP typically provided by aerobic respiration, yet the nitrogenase is irreversibly inhibited by molecular oxygen [42,43]. The Sierra Mixe aerial root mucilage was found to maintain oxygen levels below 5% at a depth of 8 mm, suggesting that the mucilage could sustain a microaerobic environment compatible with nitrogenase activity [12]. Because a similar level of oxygen depletion was observed in a 0.2% agar medium, these low oxygen levels may be achieved by mucilage-mediated reduction of oxygen diffusion from the atmosphere into the mucilage matrix. A similar depletion of oxygen levels has been reported in bacterial biofilm aggregates and by diazotrophs embedded in an exopolysaccharide pellicle, which enable nitrogen fixation in aerobic conditions [44,45]. Alternatively, oxygen levels may be actively lowered in individual diazotrophs or more generally in the mucilage matrix. For example, in both *Azotobacter vinelandii* and *Klebsiella pneumoniae* nitrogenase activity is protected from molecular oxygen by the action of an uncoupled terminal cytochrome *bd* oxidase [46,47]. Similarly, the oxygen levels of the mosquito larval midgut were recently reported to be reduced to below 5% by microbial residents with active cytochrome *bd* oxidase [48]. This suggests that a similar cytochrome *bd* oxidase-mediated mechanism could also be effective in reducing the overall oxygen levels of a closed environment with suppressed oxygen diffusion, such as maize or cereal crop root mucilage [48].

There may be other, as-yet-unrecognized mechanisms to control and reduce oxygen levels in maize mucilage, such as alternative bacterial oxidases, or even plant respiration may play a role in decreasing the oxygen concentration. Regardless of the mechanism, from merely passive diffusion restriction to active oxygen reduction, we propose that the capacity to reduce molecular oxygen levels is a critical role for the microbial community to support diazotrophs in the maize mucilage effectively.

Maintaining Low Nitrogen Levels in the Mucilage Environment

Nitrogen fixation is an energy-expensive and therefore highly regulated molecular mechanism. When nitrogen is present in the environment, diazotrophs switch off nitrogenase activity using mechanisms at the transcriptional and/or post-transcriptional level (see [49] for a review). We observed very low levels of ammonium and nitrate in the mucilage of Sierra Mixe maize (unpublished data), which is compatible with the observation that nitrogenase levels in this mucilage were very high and the nitrogenase activity was not inhibited. By spiking ^{15}N ammonium or nitrate in the mucilage, we observed a rapid incorporation of ^{15}N in plant peptides (unpublished data) suggesting that the low levels of nitrogen in the mucilage are maintained by the rapid uptake of available nitrogen by the corn aerial roots. These low nitrogen levels are likely to contribute to the enrichment of diazotrophs in the mucilage microbial community since bacteria able to fix nitrogen will have a significant advantage in such a low-nitrogen, low-oxygen, and high-carbon environment. However, questions remain about how nitrogen is transferred from bacteria to the host plant. Diazotrophs may release ammonium in the mucilage, although most diazotrophs do not waste their expensive fixed nitrogen by dispersal into the environment. Mucilage is produced by aerial roots after rain and, in the absence of rain, dries on the aerial roots. These 'wet/dry' cycles may help in releasing fixed nitrogen from the microbial community into the mucilage.

Concluding Remarks

The model of a mucilage-supported diazotrophic microbial community proposed here is based on what we have learned from a model system, the aerial root mucilage of the Sierra Mixe indigenous maize landrace. This system is striking in the extent of aerial root development and extensive exudation of mucilage, which provided the basis for these studies. However, many other cereal crops (including conventional maize, sorghum, wheat, and barley) secrete mucilage, although in much lower quantities, suggesting the possibility that similar mechanisms may be operative in other cereal crops (see Outstanding Questions).

It was previously suggested that the Sierra Mixe mucilage/diazotroph association evolved from a similar mechanism in teosinte (*Z. mays* ssp. *mexicana*) [12]. It seems likely that a similar system exists in other cereals crops. It is also striking that the mucilage characteristics (low nitrogen, high carbon, and high viscosity) closely resemble the media that microbiologists have been using to isolate diazotrophs from the environment for more than 30 years. An important caveat, however, is whether cereal crops in general produce sufficient mucilage to support this proposed model for nitrogen fixation and whether sufficient water would be available in all environments to sustain mucilage exudation.

The model proposed here suggests that there are four main functionalities required of the diazotrophic microbiota and the aerial root mucilage. Some of those functionalities, such as mucilage disassembly, use of released carbon, and lowering of oxygen levels, may exist within a single microbe. However, we speculate that this is a community function comprising several microbial species. It should be possible to test this model by analysis of metagenome and metatranscriptome sequencing of mucilage samples with active diazotrophic activity. Such studies may help to identify the critical consortium members and provide guidance in culturing microbial isolates that may be useful inoculants for crops of the future. Further studies will be required to determine whether the aerial root mucilage trait can be transferred by breeding or genetic engineering to conventional corn varieties and whether this trait is present in other cereal crops.

Outstanding Questions

Does the mucilage of cereal crops, such as wheat, sorghum, barley, and rice, comprise complex polysaccharides capable of harboring a nitrogen-fixing microbiota?

Can metagenomic and metatranscriptomic analysis provide insights into the main functionalities proposed for the diazatrophic microbiota and provide guidance in isolating members of the microbial community?

Will it be possible to identify genetic determinants of mucilage secretion and diazotrophic microbiota assembly in cereal crops and select for increased diazotrophic activity?

Will it be possible to identify individual microbes or, most likely, microbial consortia capable of fulfilling the four proposed functionalities to support nitrogen fixation?

Author Contributions

A.B.B. conceptualized and wrote the first draft of the manuscript. J-M.A. and V.C.S.P. contributed the final manuscript and to its final revisions.

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