Artificial Selection on Microbiomes to Breed Microbiomes That Confer Salt-Tolerance to Plants

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ABSTRACT

We develop a method to artificially select for rhizosphere microbiomes that confer salt-tolerance to the model grass *Brachypodium distachyon* grown under sodium-salt stress or aluminum-salt stress. In a controlled greenhouse environment, we differentially propagated rhizosphere microbiomes between plants of a non-evolving, highly-inbred plant population, and therefore only microbiomes evolved in our experiment, but the plants did not evolve in parallel. To maximize microbiome perpetuation when transplanting microbiomes between plants and thus maximize response to microbiome selection, we improved earlier methods by (a) controlling microbiome assembly when inoculating seeds at the beginning of each selection cycle; (b) fractionating microbiomes before transfer between plants to harvest, perpetuate, and select on only bacterial and viral microbiome components; (c) ramping of salt stress gradually from minor to extreme salt stress with each selection-cycle, to minimize the chance of over-stressing plants; (d) using two non-selection control treatments (e.g., non-selection microbial enrichment, null inoculation) that permit comparison to the improving fitness benefits that selected microbiomes impart on plants. Unlike previous methods, our selection protocol generated microbiomes that enhance plant fitness after only 1-3 rounds of microbiome selection. After nine rounds of microbiome selection, the effect of microbiomes selected to confer tolerance to aluminum-salt stress was non-specific (these artificially-selected microbiomes equally ameliorate sodium- and aluminum-salt stresses), but the effect of microbiomes selected to confer tolerance to sodium-salt stress was specific (these artificially-selected microbiomes do not confer tolerance to aluminum-salt stress). Plants with artificially selected microbiomes had 55-205% greater seed production compared to plants with unselected control microbiomes.

IMPORTANCE We developed an experimental protocol that improves earlier methods of artificial selection on microbiomes, then tested the efficacy of our protocol to breed root-associated bacterial microbiomes that confer salt tolerance to a plant. Salt stress limits growth and seed production of crop plants, and artificially selected microbiomes conferring salt tolerance may ultimately help improve agricultural productivity. Unlike previous experiments of microbiome selection, our selection protocol generated microbiomes that enhance plant productivity after only 1-3 rounds of artificial selection on root-associated microbiomes, increasing seed production under extreme salt stress by 55-205% after nine rounds of microbiome selection. Although we artificially selected microbiomes under controlled greenhouse conditions that differ from outdoor conditions, increasing seed production by 55-205% under extreme salt stress is a remarkable enhancement of plant productivity compared to traditional plant breeding. We describe a series of additional experimental protocols that will advance insights into key parameters that determine efficacy and response to microbiome selection.

KEYWORDS beneficial microbes, *Brachypodium distachyon*, experimental evolution, host-mediated indirect selection, microbiome selection, rhizosphere microbiome, salt stress, salt tolerance

INTRODUCTION

A challenge in plant-microbiome research is engineering of microbiomes with specific and lasting beneficial effects on plants. These difficulties of microbiome engineering derive from several interrelated factors, including transitions in microbiome function during plant ontogeny, and the complexity of microbiome communities, such as hyperdiverse rhizosphere or phyllosphere microbiomes containing countless fungal, bacterial, and viral components (1–3). Even when beneficial microbiomes can be assembled experimentally to generate specific microbiome functions that benefit a plant, microbiomes are often ecologically unstable and undergo turnover (i.e., microbiome communities change dynamically over time), for example when new microbes immigrate into microbiomes, when beneficial microbes are lost from microbiomes, or when beneficial microbes evolve new properties under microbe-microbe competition that are detrimental to a host plant.

One strategy to engineer sustainable beneficial microbiome-function uses repeated cycles of differential microbiome propagation to perpetuate between hosts only those microbiomes that have the most desired fitness effects on a host (Figure 1). Such differential propagation of microbiomes between hosts can therefore artificially select for microbiome components that best mediate stresses that impact host fitness (4–7). Only three experimental studies have used this approach so far for plants. Two studies selected on rhizosphere microbiomes of the plant *Arabidopsis thaliana* (4, 8) and both studies needed more than ten cycles of microbiome selection to generate a modest and highly variable phenotypic response in plant phenotype (e.g., increase in above-ground biomass by ≈10%; 4). A third study (9) used seven cycles of microbiome selection to generate microbiomes that significantly delayed the onset of drought symptoms of water-stressed wheat plants. We here expand on these studies to artificially select for bacterial rhizosphere microbiomes that confer salt-tolerance to the model grass *Brachypodium distachyon* (Figure 1). Our methods specifically aim to improve microbiome perpetuation between plants and to optimize response to artificial microbiome selection by controlling microbiome assembly when inoculating seeds; using low-carbon soil to enhance host-control exerted by seedlings during initial microbiome assembly and early plant growth; harvesting and perpetuating microbiomes that are in close physical contact with plants; short-cycling of microbiome generations to select for microbiomes that benefit seedling growth; ramping of salt-stress between selection-cycles to minimize the chance of either under-stressing or over-stressing plants.

The Logic of Host-Mediated Indirect Artificial Selection on Microbiomes. To optimize microbiome selection experiments, we found it useful to conceptualize the process of microbiome selection within a host-focused quantitative-genetic framework (6), rather than within a multi-level selection framework preferred by Swenson *et al* (4; "artificial ecosystem selection"; see also 10). Both frameworks capture the same processes (i.e., neither framework is wrong), but a host-focused quantitative-genetic framework is more useful to identify factors that can be manipulated to increase efficacy of microbiome selection. First, because microbiome selection aims to shape a fitness component of the host-plant (e.g., stress tolerance), and because it is typically easier to measure plant phenotypes rather than measure microbiome properties, selection is indirect: Microbiomes are not measured directly, but microbiomes are evaluated indirectly by measuring host performance. Indirect selection is an established breeding technique that can be used when the target trait is difficult or costly to measure (11), as is the case also for microbiome traits, compared to the ease of measuring a host phenotype that is dependent on microbiome properties. The efficacy of indirect selection depends on strong correlations between microbiome and host traits; indirect microbiome selection should therefore be more efficient if such correlations can be maximized experimentally, for example by controlling ecological priority-effects during initial microbiome assembly (12–15), or by increasing host-control over microbiome assembly and persistence (14, 16). Second, because a typical host likely experienced a long history of evolution to monitor and manipulate its microbiomes (a process called *host control*; 16–19), indirect microbiome selection uses the host as a kind of "thermostat" to help gauge and adjust the "temperature" of its microbiomes, then propagate desired microbiomes between hosts (Figure 1). Based on theory (5, 6, 20), such host-mediated indirect selection on microbiomes can be easier than direct selection on microbiomes, particularly with host species that exert strong host-control over assembly and stability of their microbiomes (6, 13, 14, 21).

Definition of Microbiome Engineering & Microbiome Selection. Microbiome engineering by means of differential microbiome propagation (Figure 1) alters microbiomes through both ecological and evolutionary processes. Ecological processes include changes in community diversity, relative species abundances, or structure of microbe-microbe or microbe-plant interaction networks. Evolutionary processes include extinction of specific microbiome members; allele frequency changes, mutation, or gene transfer between microbes; and differential persistence of microbiome components when differentially propagating microbiomes at each selection cycle. These processes can be interdependent (e.g., in the case of eco-evolutionary feedbacks; 22, 23), and some processes can be called either ecological or evolutionary (e.g., loss of a microbe from a microbiome can be viewed as evolutionary extinction, or as an outcome of ecological competition), but for the design of a microbiome-selection protocol, it is useful to think about ecological processes separately from evolutionary processes. Microbiome-selection protocols aim to

maximize changes in the genetic makeup of microbiomes through differential microbiome propagation (Steps 3&4 in Figure 1), for example by optimizing microbiome transmission during microbiome transplanting between hosts, or by optimizing microbiome re-assembly after such transfers (e.g., by facilitating ecological priority effects at host inoculation). Although both evolutionary and ecological processes alter genetic makeup of microbiomes during each propagation cycle (Figure 1), as a shorthand, we refer here to the changes resulting from host-mediated indirect selection on microbiomes as 'microbiome response' due to 'microbiome selection'.

RESULTS

Artificially-Selected Microbiomes Confer Increased Salt-Tolerance to Plants. Figure 2 shows the changes in relative plant fitness (above-ground dry biomass) during eight rounds of differential microbiome propagation. Relative to Fallow-Soil Control (non-selection enrichment) treatment and Null Control treatment, selected microbiomes confer increased salt-tolerance to plants after only 1-3 selection-cycles, for both the sodium-stress (Figure 2a&c) and the aluminum-stress treatments (Figure 2b&d). Relative to Fallow-Soil Control plants, artificially selected microbiomes increase plant fitness by 75% under sodium-sulfate stress ($p<0.001$), and by 38% under aluminum-sulfate stress ($p<0.001$). Relative to Null Control plants, selected microbiomes increase plant fitness by 13% under sodium-sulfate stress, and by 12% under aluminum-sulfate stress. Although repeated rounds of differential microbiome propagation improved plant fitness between successive microbiome generations (particularly relative to the Null Controls; Figures 2c&d), interactions between treatment and generation were not statistically significant (see Supplemental Material Results). This implies that fitness-enhancing effects of microbiomes from selection lines therefore were realized after one or a few rounds of microbiome selection (e.g., Figure 2c&d), and there was insufficient statistical support that, under the gradually increasing salt stress, any additional rounds further resulted in greater plant biomass of selection-lines relative to control-lines. However, because plants were exposed to increasingly greater salt stresses in later generations (Figures 2e&f, Supplemental Material Results), selected microbiomes of later generations helped plants tolerate more extreme salt stresses.

The phenotypic effect on plants due to the evolving microbiomes fluctuated during the eight rounds of differential microbiome propagation (Figures 2a-d). Such fluctuations can occur in typical artificial selection experiments (24), but fluctuations may be more pronounced when artificially selecting on microbiomes (25) because additional factors can contribute to between-generation fluctuations. Specifically, across the eight selection cycles in our experiment, the observed fluctuations could have been due to (i) uncontrolled humidity changes and correlated humidity-dependent water-needs of plants (humidity was not controlled in our growth chamber), consequently changing the effective salt-stresses; (ii)

the strong ramping of salt-stress during the first five selection-cycles, possibly resulting in excessively stressed plants in Generations 4&5 (see discussion in Supplemental Material Methods); (iii) random microbiome changes ("microbiome drift") and consequently random microbe-microbe interactions; or (iv) other such uncontrolled factors. The fluctuations in plant fitness are most prominent during the first five selection-cycles (Figures 2a-d) when we increased salt-stress 2- to 5-fold between generations and when humidity varied most in our growth chamber (see Supplemental Material Methods), whereas fluctuations were less pronounced during the last three generations when we changed salt-stress only minimally and humidity was relatively stable. These observations are consistent with known responses of *B. distachyon* to environmental stresses (26), predicting that artificial selection on microbiomes conferring salt-tolerance to plants should be most efficient under experimental conditions that rigorously control soil moisture, salt-stress, humidity, and plant transpiration.

Effect of Artificially Selected Microbiomes on Seed Production. In the last microbiome-generation after a ninth microbiome-selection cycle (Generation 9), we grew plants for 68 days to quantify the effect of our artificially-selected microbiomes on seed production. We also added one control treatment, *Solute-Transfer Control* (*Solute Control*), to help elucidate some of the mechanisms underlying the salt-tolerance-conferring effects of selected microbiomes on seed production (Figure 3). In Solute Control treatments, we eliminated with 0.2μm filters live cells from the harvested microbiomes in the selection lines, to test the growth-enhancing effects of root exudates and viruses that may be co-propagated with bacterial microbiomes in the selection-lines. Plants receiving these bacteria-free, filtered solutes had (i) significantly poorer seed-production compared to plants that received these same solutes together with the live bacterial microbiomes ($p<0.02$ for sodium-stress treatment; $p<0.05$ for aluminum-stress treatment; Supplemental Material Results); and (ii) seed production that was comparable to plants from Null Control treatments ($p>0.7$ for sodium-stress treatment; $p>0.25$ for aluminum-stress treatment; Supplemental Material Results). These findings indicate that any plant exudates or viruses co-propagated with bacterial microbiomes did not account for the salt-tolerance-conferring effects of selected microbiomes, and that any co-transplanted solutes (e.g., root exudates) and any co-propagated viruses affected plant growth like Null Control treatments (i.e., no exudates, no viruses).

Specificity Test by Crossing Evolved SOD- and ALU-Microbiomes with SOD- and ALU-Stress. In the *Cross-Fostering* Control of the last microbiome-generation, we crossed harvested microbiomes from the sodium-stress (SOD) and aluminum-stress (ALU) selection-lines with the two types of salt-stress in soil, to test specificity of the salt-ameliorating effects of the microbiomes (Figure 4; Table S2). The effect of microbiomes selected to confer tolerance to aluminum-sulfate appears non-specific (aluminum-selected

microbiomes appear to confer equal tolerance to both sodium- and aluminum-sulfate stress; $p>0.5$, Figure 4), but the effect of bacterial microbiomes selected to confer tolerance to sodium-sulfate appears specific (sodium-selected microbiomes confer less tolerance to aluminum-sulfate stress; $p<0.002$, Figure 4).

DISCUSSION

Our study aimed to improve the differential microbiome-propagation scheme that was originally developed by Swenson *et al* (4), then test the utility of our improved methods by artificially selecting on microbiomes to confer salt-stress tolerance to plants. Swenson's original whole-soil community propagation scheme failed to generate consistent benefits for plant growth, and growth enhancement due to putatively selected communities was overall minor when averaged across all propagation-cycles (average of ≈10% growth enhancement). To address these problems, we adopted in our experiment ideas from quantitative genetics, microbial ecology, and host-microbiome evolution to optimize steps in our microbiome-propagation protocol (Figure 1), with the aim to improve perpetuation of beneficial microbiomes. Specifically, our methods aimed to (a) facilitate ecological priority-effects during initial microbiome assembly (13, 14, 21), thus increasing microbiome-inheritance by steering the initial recruitment of symbiotic bacteria into rhizosphere microbiomes of seedlings; (b) propagate microbiomes harvested from within the sphere of host-control (i.e., microbiomes in close physical proximity to roots), whereas Swenson *et al* (4) and likewise Panke-Buisse *et al* (8) harvested also microbes from outside the sphere of host control; (c) enhance carbon-dependent host-control of microbiome-assembly and of microbiome-persistence by using low-carbon soil (1, 6, 27, 28); and (d) gradual ramping of salt-stress between selection-cycles to minimize the chance of either under-stressing or over-stressing plant. Without additional experiments, it is not possible to say which of these experimental steps was most important to increase response to microbiome selection. Because Jochum *et al* (9) succeeded at artificially selecting for microbiomes that confer drought tolerance to wheat grown in high-carbon soil, either low-carbon soil may not be essential for plant-mediated microbiome selection, contrary to our assumption; or high-carbon soil may facilitate microbiome selection of fungal components because Jochum *et al* (9) propagated between generations both bacterial and eukaryote rhizosphere components.

Compared to two earlier experiments of host-mediated microbiome selection by Swenson *et al* (4) and likewise Panke-Buisse *et al* (8) , our selection scheme appears to generate more pronounced and more stable effects on plant phenotype as a result of host-mediated microbiome selection. Except for the initial two selection-cycles (Figures 2a-d), our selected microbiomes consistently outperformed in subsequent selection-cycles the non-selected microbiomes of the control conditions. In contrast, for example, Swenson *et al*'s (4) experiments sometimes resulted in selected microbiomes that were outperformed by control

microbiomes. Our methods may have generated more stable microbiome-effects because (a) only bacteria, but no fungi were propagated between generations (Swenson *et al* suspected fungal disease as cause of occasional devastation of plant populations); (b) we conducted our experiment in a more stable growth environment; and (c) we selected for microbiomes conferring specific benefits (salt-tolerance), rather than the non-specific, general-purpose beneficial microbiomes selected by Swenson *et al* (4) and Panke-Buisse *et al* (8). After only 1-3 selection cycles, our selected microbiomes consistently outperformed the control microbiomes, with averages of 75% (SOD) and 38% (ALU) growth improvement relative to Fallow-Soil Controls; and 13% (SOD) and 12% (ALU) growth improvement relative to Null Controls (Figures 2a-d). Most importantly, when quantifying plant fitness by total seed production in the final Generation 9, plants with selected microbiomes outperformed Fallow-Soil Controls, Null Controls, and Solute Controls by 120-205% (SOD) and 55-195% (ALU) (Figure 3). Although we achieved these results under controlled greenhouse conditions that are very different from outdoor conditions, this seems a remarkable enhancement of plant productivity compared to traditional plant breeding.

An interesting result is that microbiomes selected to benefit growth of plants during the early vegetative phase (biomass of ≈4-week-old plants, well before flowering; Figure 2) generated microbiomes that enhanced plant fitness also during the reproductive phase by increasing seed set of 10-week-old plants (Figure 3). Rhizosphere microbiomes of grasses can change significantly during plant ontogeny (29), and therefore microbiomes selected to serve one function such as early growth may not necessarily optimize other functions such as seed set. The finding that microbiome selection to promote early growth (Figure 2) also promotes increased seed set (Figure 3) therefore could imply that (a) seed set is intrinsically tied to optimal early growth in *B. distachyon*, possibly by accelerating the timing of flowering; (b) some of the same bacteria benefitting plants during the early vegetative phase also benefit plants during the reproductive phase, despite overall microbiome changes during plant ontogeny; and (c) microbiome-selection experiments aiming to increase seed productivity do not necessarily have to select on seed set as a measured phenotype, but can shorten each selection cycle by selecting on other phenotypes measurable during early vegetative growth.

Because Jochum *et al*'s (9) and our experiments were the first systematic attempts to improve the methods of Swenson *et al* (4), we predict that it should be possible to further optimize protocols of differential microbiome propagation. Microbiome selection therefore could emerge as a novel tool to elucidate microbiome-functions in controlled laboratory environments, and possibly also in those natural environments that allow control of key parameters affecting microbiome harvest, microbiome transfer, and microbiome inheritance. Such optimization of microbiome selection should ideally be informed by

metagenomic analyses of experimental contrasts (e.g., comparison of microbiomes selected to confer tolerance to either sodium-stress or aluminum-stress) and by time-series analyses across microbiome-propagation cycles, to identify candidate microbes and microbial consortia important in mediating stresses.

Additional Experiments to Improve Methods of Microbiome Selection. To expand on our methods of artificial microbiome selection, we outline here a series of additional experiments that should generate insights into key parameters that determine efficacy of microbiome selection. Arias-Sánchez *et al* (7), Xie *et al* (30), Chang *et al* (31, 32), and Sánchez *et al* (33) recently summarized criteria for microbiome-selection experiments that are not host-mediated (e.g., selection on CO_2 emission by a microbiome, in absence of a plant host); Lawson *et al* (34) summarized protocols for engineering any kind of microbiome (e.g., using bottom-up and top-down design criteria); Henry *et al* (35), Arora *et al* (36), and Henry & Ayroles (37) developed methods for host-mediated microbiome selection using *Drosophila* as a host; and we focus below on methods of host-mediated microbiome selection to improve performance of a plant host. Because host-mediated microbiome selection leverages traits that evolved to recruit and control microbiomes (so-called host control; 6, 19, 38), the first four experiments outlined below explore whether factors promoting strong microbiome control by a plant host could improve efficacy of microbiome selection:

(1) Artificial microbiome selection on endophytic vs. rhizosphere microbiomes: Microbiomes internal to a host (e.g., endophytic microbes of plants) require some form of host infection, and therefore could be under greater host control than external microbiomes, such as rhizoplane or rhizosphere microbiomes. Consequently, under stresses that are mediated by host-controlled microbes, it may be easier to obtain a response to microbiome selection when targeting selection on endophytic microbiomes. This prediction can be tested in an experiment that compares, in separate selection lines, the responses to microbiome selection when harvesting and propagating only endophytic microbiomes versus only rhizosphere microbiomes. This prediction may not hold for stresses that require stress-mediation by microbes in the external microbiome compartment of roots (e.g., microbes that detoxify toxins, such as aluminum, before they enter the root and then affect the plant negatively; for example microbes that chelate toxins external to the plant in the rhizosphere, 39); however, this prediction about a key role of host control for the efficacy of microbiome selection should hold for many other stresses that are mediated by microbes that a plant permits to enter into the endophytic compartment.

(2) Microbiome selection in two genetic backgrounds differing in host-control: A second approach to test for the role of host control is to compare microbiome selection in two different host-genotypes, such as two inbred strains of the same plant species. For example, different host genotypes may recruit into symbiosis different kinds of microbes (40). Such differences in host-controlled microbiome recruitment

could result in differences in microbiome selection, and a microbiome artificially-selected within one host-genotype to improve one particular host trait may produce a different phenotypic effect when tested in a different host genotype.

(3) *Varying host control by varying carbon-content in soil*: A third approach to test host control is to compare efficacy of microbiome selection in low- versus high-carbon soil. Microbial growth in some soils is limited by carbon, and many plants therefore regulate their soil-microbiomes by carbon exudates (41). We therefore hypothesized that a low-carbon soil (like the carbon-free soil in our experiment) may facilitate host control and consequently also microbiome selection. This hypothesis remains to be tested, for example in a microbiome-selection experiment contrasting response to selection when using soils with different carbon content. Because Jochum *et al* (9) recently showed that it is possible to artificially select for microbiomes that confer drought tolerance to wheat grown in high-carbon soil, low-carbon soil may not be essential for plant-mediated microbiome selection, but low-carbon soil could be a facilitating condition.

(4) *Manipulating resource-limited host control by varying seed size*: A fourth approach to test host control could be to compare the efficacy of microbiome selection between plant species with large seeds versus small seeds (e.g., *Brachypodium* versus *Arabidopsis*), or between seedlings of the same species grown from small versus large seeds. A germinating seed has to allocate resources to above-ground growth to fix carbon and to below-ground growth to access nutrients and water, and seedlings growing from resource-rich large seeds therefore may be better able to allocate resources to manipulate microbiomes effectively, for example by root exudates. If such resource-allocation constraints exist for young seedlings, this could explain why our microbiome-selection experiment with *B. distachyon* appears to have generated stronger and faster response to microbiome selection compared to other such experiments with *Arabidopsis thaliana* (4, 8).

(5) *Propagation of fractionated vs. whole microbiomes*: Experimental microbiome propagation between host generations can be complete [all soil-community members are propagated between hosts, as in Swenson *et al* (4), Panke-Buisse *et al* (8), Jochum *et al* (9)] or microbiomes can be fractionated by excluding specific microbial components, as in our protocol where we propagated only organisms of bacterial or smaller sizes. We used fractionated microbiome-propagation because (a) we were more interested in elucidating contributions to host fitness of the understudied bacterial components than the fungal components (e.g., mycorrhizal fungi), and (b) fractionation simplifies analyses of the microbiome-responses to selection (e.g., bacterial microbiome components, but not necessarily fungal components, need to be analysed with metagenomic techniques). However, because fungal components and possible synergistic fungal-bacterial interactions cannot be selected on when using our fractionated microbiome-propagation scheme, we hypothesized previously (6) that selection on fractionated

microbiomes may show attenuated selection responses compared to selection on whole microbiomes. This can be tested in an experiment comparing the response to microbiome selection when propagating fractionated versus whole microbiomes, for example by using different size-selecting filters.

(6) *Propagation of mixed vs. un-mixed microbiomes*. When propagating microbiomes to new hosts, it is possible to propagate mixed microbiomes harvested from different hosts, or only un-mixed microbiomes. Mixed vs. un-mixed propagation schemes therefore represent two principal methods of microbiome selection (4–6, 42, 43). Compared to un-mixed propagation, mixed propagation generated a faster response to microbiome selection for microbiomes propagated in vitro in the absence of a host (43), but the respective advantages of mixed versus un-mixed propagation have yet to be tested for host-associated microbiomes, such as the rhizosphere microbiomes studied here. Mixed propagation may be superior to un-mixed propagation, for example if mixing generates novel combinations of microbes with novel beneficial effects on a host (6), may merge separate networks of microbes into a superior compound network (so-called community-network coalescence; 42, 44), or may generate novel microbial interactions that increase microbiome stability (13).

(7) *Microbiome diversity of the starter inoculum*. In our salt-stress experiment, we aimed for a highly diverse starter microbiome to inoculate all pots of Generation 0, but we did not specifically try to include bacteria from sources that are most likely to include microbes that confer salt tolerance to plants. Could inclusion of microbiomes harvested from grasses growing naturally in salty soil have improved the diversity of bacteria in the starter inoculum, and thus increased the response to microbiome selection in our experiment? Comparison of starter inocula harvested from plants growing naturally in salty versus non-salty soils may be able to address this question.

MATERIALS AND METHODS

We developed our microbiome-selection protocol between 2011-2014 in a series of pilot experiments, conducted the microbiome-selection experiment reported here between January-October 2015, then disseminated our protocol via bioRxiv in 2016 (45) to facilitate teaching of workshops on microbiome selection. We describe here our experimental protocols, and a separate report (Edwards *et al* in preparation) will describe the metagenomic analyses complementing the protocols and phenotypic results reported here.

Maximizing microbiome perpetuation. To select for microbiomes that confer salt-tolerance to plants, we used a differential host-microbiome co-propagation scheme as described in Swenson *et al* (4), Mueller *et al* (46), and Mueller & Sachs (6), but improved on these earlier selection schemes by (i) maximizing evolutionary microbiome changes stemming from differential propagation of whole microbiomes at Step 3 in Figure 1, while (ii) minimizing some, but not all, ecological microbiome changes that can occur at any of the steps in a selection cycle (e.g., we tried to minimize uncontrolled microbe-community turnover). In

essence, our protocol aimed to maximize microbiome perpetuation (i.e., maximize inheritance of key microbes). To increase microbiome inheritance, we added protocol steps of known techniques, most importantly (i) facilitation of ecological priority-effects during initial microbiome assembly (21), thus increasing microbiome-inheritance by controlling in each selection-cycle the initial recruitment of symbiotic bacteria into rhizosphere microbiomes of seedlings; and (ii) low-carbon soil to enhance carbon-dependent host-control of microbiome assembly and microbiome persistence (1, 6, 27, 28). Theory predicts that any experimental steps increasing fidelity of microbiome perpetuation from mother microbiome to offspring microbiome should increase the efficacy of microbiome selection (6, 30, 35, 47).

Maximizing microbiome heritability. In each microbiome-propagation cycle (microbiome generation), we inoculated surface-sterilized seeds taken from non-evolving stock (inbred strain Bd3-1 of the grass *Brachypodium distachyon*; 48), using rhizosphere bacteria harvested from roots of those plants within each selection line that exhibited the greatest above-ground biomass (Figure 1). Microbiome selection within the genetic background of an invariant (i.e., highly inbred) plant genotype increases *microbiome heritability*, defined as the proportion of overall variation in plant phenotype that can be attributed to differences in microbiome-encoded genetic effects on plants. By keeping plant genotype invariant, microbiome heritability increases because a greater proportion of the overall plant-phenotypic variation in a selection line can be attributed to differences in microbiomes. This increases an experimenter's ability to identify association with a desired microbiome (4), enhancing reliability of the plant phenotype as indicator of microbiome effects, and thus increasing efficacy of indirect selection on microbiomes.

Harvesting rhizosphere microbiomes & selection scheme. Each selection line consisted of a population of eight replicate plants, and each selection treatment had five replicate selection lines (i.e., 40 plants total per treatment). To phenotype plants on the day of microbiome harvesting, we judged above-ground growth visually by placing all eight plants of the same selection line in ascending order next to each other (Figure S3), then choosing the two largest plants for microbiome harvest. For all plants, we cut plants at soil level, then stored the above-ground portion in an envelope for drying and weighing. For each plant chosen for microbiome harvest, we extracted the entire root system from the soil, then harvested rhizosphere microbiomes immediately to minimize microbiome changes in the absence of plant-control. Root structures could be extracted whole because of a granular soil texture (Profile Porous Ceramic soil), with some loss of fine roots. Because we were interested in harvesting microbiomes that were in close association with roots, we discarded any soil adhering loosely to roots, leaving a root system with few firmly attached soil particles. We combined the root systems from the two best-growing plants of the same selection line, and harvested their mixed rhizosphere microbiomes by immersing and gently shaking the roots in the same salt-nutrient buffer that we used also to hydrate soils (details in Supplemental Material Methods). Combining root systems from the two best-growing plants generated a so-called *mixed microbiome* harvested from two

mother-rhizospheres, which we then transferred within the same selection line to all eight offspring-plants (i.e., germinating seeds) of the next microbiome generation (Figure 1).

Microbiome fractionation with size-selecting filters before microbiome propagation. To simplify future metagenomic analyses from propagated microbiomes, we used 2µm filters (details in Supplemental Material Methods) to filter microbiomes harvested from rhizospheres of mother-plants, thus capturing only bacteria (and possibly also viruses) for microbiome propagation to the next microbiome-generation, but eliminating from propagation any larger-celled soil-organisms (i.e., we excluded all eukaryote organisms in soil, including fungi). This fractionation step distinguishes our methods from those of Swenson *et al* (4), Panke-Buisse *et al* (8), and Jochum *et al* (9), all of which transferred between pots all organism living in soil (including algae, nematodes, protozoans, fungi, etc). Plant phenotypic changes in these previous experiments were therefore not necessarily due to changing microbiomes, but possibly to eukaryotes that were co-propagated with microbiomes, whereas we transferred only bacteria and viruses between microbiome generations to rule out any confounding effects of co-propagated eukaryotes.

Salt-stress treatments and experimental contrasts. Using different selection-lines, we selected for beneficial microbiomes conferring salt-tolerance to either sodium-sulfate [Na_2SO_4] or aluminum-sulfate [$Al_2(SO_4)_3$]. Such an experimental contrast of two treatments (here, two salt stresses) enables an experimenter to (i) compare evolving microbiomes using metagenomic time-series analyses, (ii) identify candidate microbes (indicator taxa) that differ between salt treatments and that may therefore confer salt-tolerance to plants, and (iii) test the specificity of beneficial effects of evolved microbiomes in a cross-fostering experiment (see below).

Control treatments. To evaluate the effects of selection treatments, we included two non-selection control treatments. In *Null Control*, we did not inoculate germinating seeds with any microbiomes, but microbes could enter soil from air, as was the case also for all other treatments. In *Fallow-Soil Microbiome-Propagation Control*, we harvested microbiomes from fallow soil (no plant growing in a pot; microbiomes were harvested from root-free soil), then propagated the harvested microbiomes to a pot with sterile fallow soil of the next microbiome generation. Specifically, each microbiome harvested from fallow soil was split, one part was propagated to sterile fallow soil to start the next microbiome generation, and another part of the same microbiome was applied to seeds planted in sterile soil, to test the effect of such fallow-soil microbiomes on the growth of plants (details in Supplemental Material Methods). Fallow-Soil Control is a non-selection treatment because a microbiome is transferred from exactly one pot in the previous generation to one pot in the next generation, resulting in enrichment (49) of microbes that proliferate under the specific salt conditions in soil, but in the absence of higher-level microbiome selection that, in the selection treatment, selectively perpetuate growth-promoting microbiomes while discarding inferior microbiomes (i.e., there is no such discarding of inferior microbiomes in the Fallow-Soil Control treatment).

Number of selection cycles. Our complete experiment involved one baseline Generation (Generation 0, Table S1) to establish initial microbiomes in replicate pots; eight rounds of differential microbiome-propagation (Generations 1-8, Table S1); and one final round (Generation 9, Table S2) to evaluate the effects of the artificially-selected microbiomes on seed-set, for a total of 10 microbiome-generations.

Ramping of salt stress. We increased salt stresses gradually during the selection experiment, by (i) increasing between generations the molarity of the water used to hydrate dry soil before soil-sterilization and planting (Supplemental Material Methods); and (ii) increasing correspondingly also the molarity of the water that was added regularly to pots of growing plants to keep soils hydrated (Supplemental Material Methods). Over the 10 generations, sodium-sulfate molarity in sodium-stress treatments increased from 20 millimolar (mM) to 60 mM, and aluminum-sulfate molarity in aluminum-stress treatments increased from 0.02 mM to 1.5 mM (Supplemental Material Methods). The salt stresses of the baseline generation were chosen because, in pilot experiments, these stresses caused minimal delays in germination and growth compared to unstressed plants (see Supplemental Material Methods). We did not pre-plan any maximum salt stresses that we wanted to reach via ramping within the 10 generations of microbiome propagation, because the salt stresses were increased judiciously each generation such that the plants would not be overstressed (because then beneficial microbiomes would not be able to ameliorate severe salt stresses) nor plants would be understressed (and plants would then not need the help of beneficial microbiomes). The logic of increasing salt stresses stepwise between generations, and once decreasing salt stresses between Generations 5 & 6 when plants seemed overstressed (Figure 2), is explained in the Supplemental Material Methods under the subheading Soil Hydration & Salt-Stress Treatments.

Diversity of starter microbiome for baseline Generation 0. We prepared a single, well-mixed bacterial microbiome batch to inoculate all pots of the initial baseline Generation 0, combining bacterial microbiomes from several rhizosphere sources to maximize the bacterial diversity of this starter inoculum. We used 2µm Whatman™ filters to filter bacterial communities from root systems of three local grass species (*Bromus* sp., *Andropogon* sp., *Eragrostis* sp.); and from root-systems of *B. distachyon* Bd3-1 plants used in earlier experiments (see Supplemental Material Methods). We combined microbiomes from several sources in the hope of capturing a great diversity of bacteria, and we included microbiomes harvested from Bd3-1 roots in order to capture bacterial taxa that may be readily recruited by *B. distachyon* into its rhizosphere. This diverse starter microbiome changed during Generation 0 through the aforementioned ecological processes once associated with a plant. The resulting variation in microbiomes between experimental replicates contributed to the variation in plant growth that we used for indirect selection on microbiome properties.

Statistical Analyses: Plant Biomass, Generations 1-8: We performed all analyses in R v3.3.1. We assessed differences in above-ground plant biomass (dry weight) among treatments of Generations 1-8 by fitting the data to a generalized linear mixed model with a gamma error distribution. Statistical significance

in the GLMMs was assessed with likelihood ratio tests and Tukey tests employed for post-host comparisons of treatment means (more details in Supplemental Material Statistical Analyses).

Statistical Analyses: Total Seed Weight, Generation 9: Because plants were severely salt-stressed in Generation 9 and many plants therefore did not flower or only produced very few seeds, the distribution of data was not normal (Figure S5 top-left). We attempted several data-transformations to achieve approximate normality, but none of these transformations generated a distribution that approximated normality (Figures S5b-d). We therefore used Kruskal-Wallis tests for non-parametric evaluation of differences between treatments in Generation 9; and we used Mann-Whitney U-tests for non-parametric post-hoc comparisons between treatment means, correcting p-values using the false discovery rate. All tests were two-tailed with alpha=0.05 (more details in Supplemental Material Statistical Analyses).

Availability of data, materials, and methods. All data are available in Supplemental Tables S1 & S2. All methods are described in detail in the Supplemental Material Methods.

SUPPLEMENTAL MATERIAL

File 1: Supplemental Material Methods

File 2: Supplemental Material Statistical Analyses

File 3: Supplemental Material Results

File 4: Supplemental Table S1

File 5: Supplemental Table S2

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AUTHOR CONTRIBUTIONS

UGM, DLD, KB, and TEJ developed the plant methods; UGM developed the microbial and microbiome-selection methods; TEJ contributed equipment and Bd3-1 seeds; UGM, MRK, and ALC conducted experiments; ALC and MRK recorded all data (dry weights) blindly; JAE, CCS, and CCF analyzed the data and designed figures; UGM led the writing of the manuscript.

COMPETING INTERESTS

No competing interests.

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FIGURES

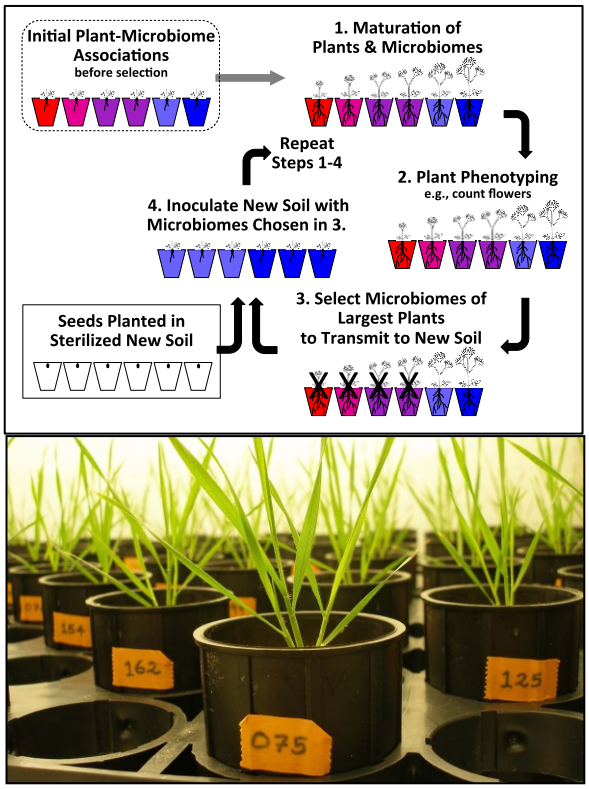


Figure 1. Host-mediated artificial selection on microbiomes. Top: Method of differential microbiome propagation to impose artificial selection on rhizosphere microbiomes (modified from 6). The host plant does not evolve because this method harvests microbiomes from mature plants and propagates these microbiomes to sterilized seeds planted in sterilized soil (Step 4), but seeds are taken each cycle from a non-evolving source (stored seeds). The method imposes indirect selection on microbiomes because microbiome properties are not measured directly; instead, microbiome effects are estimated indirectly by measuring host fitness (e.g., plant biomass), and host fitness is therefore used as an indicator to infer association with rhizosphere microbiomes that benefit a plant. Both evolutionary and ecological processes can alter microbiomes at each Step in the cycle (see text), but at Steps 3 & 4 in each cycle, experimental protocols aim to maximize evolutionary changes stemming from differential microbiome propagation. **Bottom:** Experimental plants of the model grass *Brachypodium distachyon* shortly before harvesting of rhizosphere microbiomes for differential microbiome propagation. Photo by UGM.

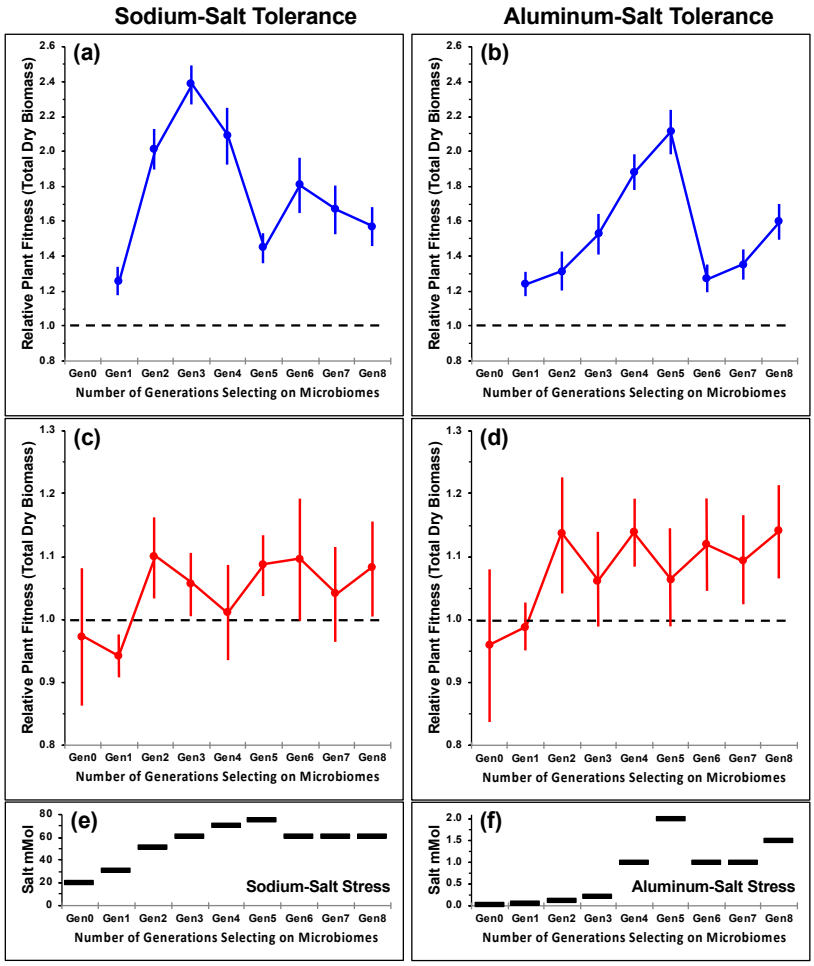


Figure 2. Artificial selection on microbiomes to generate microbiomes that confer salt-tolerance to plants, Microbiomes were artificially selected in two concurrent experiments under either sodium-salt stress (left column) or aluminum-salt stress (right column). After microbiome inoculation of plants in the baseline generation (Gen0), microbiomes were propagated differentially for 8 selection cycles (generations = Gen), using the microbiome propagation scheme in Figure 1. Two salt stresses, sodium-sulfate stress **(a,c,e)** and aluminum-sulfate stress **(b,d,f)**, were imposed in parallel in different lines of microbiome selection. Fitness of plants receiving artificially-selected microbiomes is shown in **a-d** relative to two non-selected control treatments: In *Fallow-Soil Microbiome-Propagation Control*, microbiomes were harvested from fallow soil (soil in pot with no plant), then propagated to sterile fallow soil of the next microbiome generation. In *Null Control*, plants did not receive microbiome inocula, but microbes could "rain in" from air, as in all treatments. Horizonal dashed lines in **a-d** indicate the threshold above which plants given selected microbiomes had higher relative fitness in comparison the control plants, relative to Fallow-Soil Control plants (**a&b**) and relative to Null Control plants (**c&d**). Each selection treatment had 5 selection lines (8 plants/line), and the error bars show the standard deviation of the 5 averages of these 5 selection lines. Salt stresses were increased between selection-cycles (**e&f**), starting with minor salt stresses, increasing gradually to minimize the chance of overstressing the plants, but decreasing a salt stress if plants seemed overstressed (details in Supplemental Material Methods). Because of the increasing salt stresses (**e&f**), selected microbiomes enabled plants to cope with more severe stresses, and therefore had stronger fitness-enhancing effects on plants, in later generations. **Findings:** Relative to Fallow-Soil Control treatments, selected microbiomes increase plant fitness by 75% under sodium-sulfate stress (**a**), and by 38% under aluminum-sulfate stress (**b**). Relative to Null Control treatments, selected microbiomes increase plant fitness by 13% under sodium-sulfate stress (**c**), and by 12% under aluminum-sulfate stress (**d**).

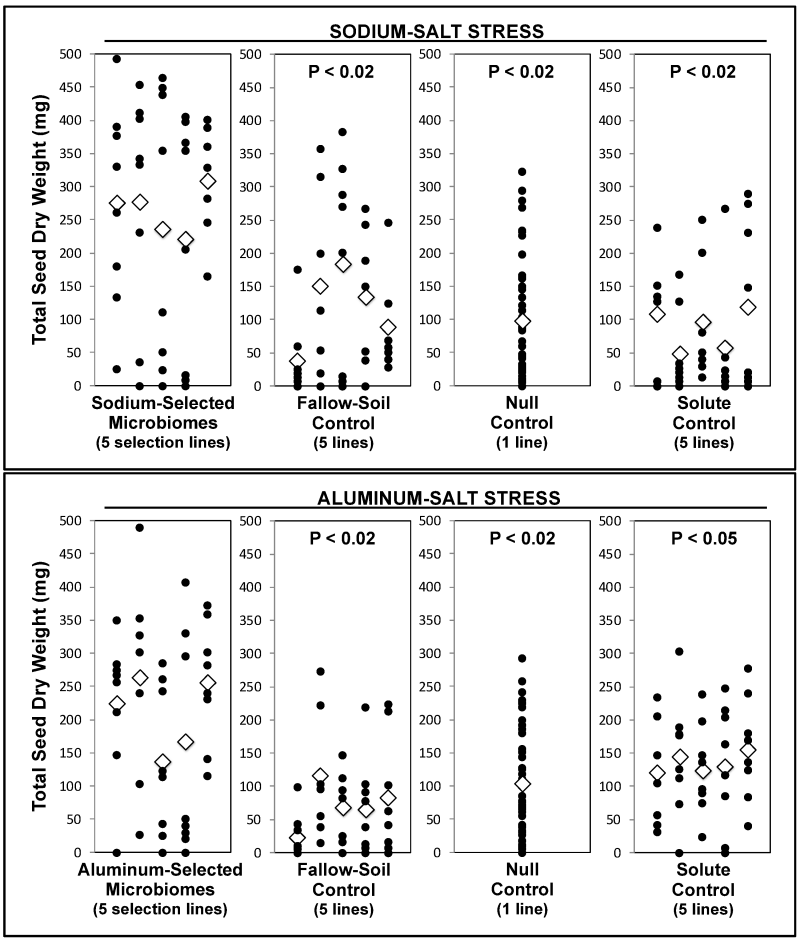
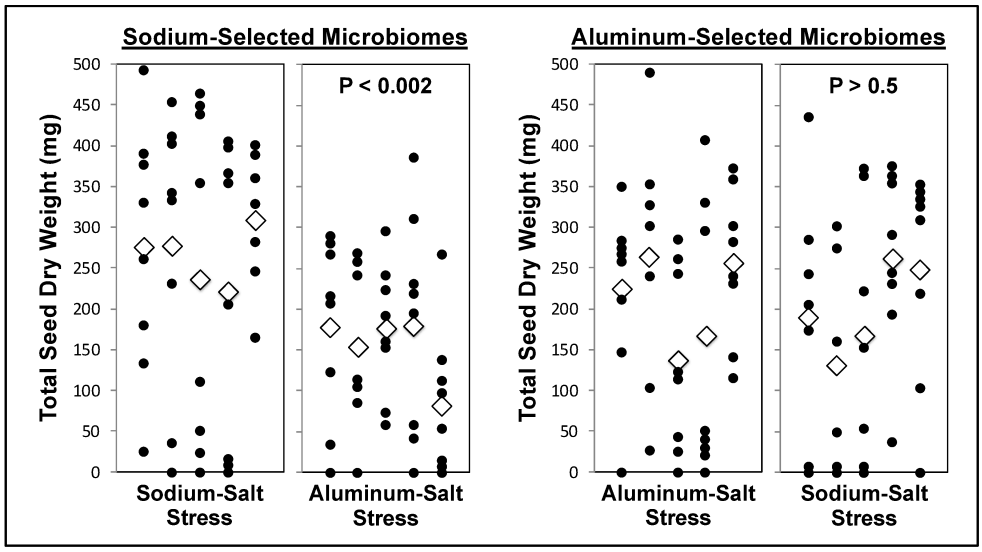


Figure 3. Artificially selected microbiomes increase seed production under salt stress. At the end of our experiment after a ninth selection cycle (Generation 9), plants were grown to seed for 68 days, to test whether rhizosphere microbiomes selected to increase above-ground biomass of pre-flowering plants generated microbiomes that also enhance seed production. Total seed dry weight is plotted as a black dot for each plant; plants of the same selection line are plotted vertically above each other; and the average for each line is plotted as a diamond. Overlapping datapoints are adjusted here minimally to separate such datapoints and visualize all datapoints. In addition to *Fallow-Soil Control* and *Null Control* used in Generations 1-8 (Figure 2), *Solute Control* was added in Generation 9. In Solute Control, selected bacterial microbiomes harvested from rhizospheres were filtered to remove also all bacterial components, to test for any growth-enhancing effects of viruses and solutes (e.g., plant hormones exuded into soil) that are unavoidably co-propagated with any harvested rhizosphere microbiome. All controls are significantly different from the corresponding selection treatment (leftmost panel); P-values are shown above each control, and P-values are corrected using the false discovery rate for post-hoc comparisons (Supplemental Material Results). **Findings:** Plants were salt-stressed because many plants never produced seeds (or few seeds; see also Figure S5 top-left), whereas essentially all plants would produce many seeds under stress-free conditions. Artificially selected microbiomes helped plants cope with these salt stresses, because plants that received selected microbiomes outperformed plants of all three control treatments, including Solute-Control plants (indicating that selected bacterial microbiomes conferred salt-tolerance to plants, rather than any co-propagated viruses). Seed production of Solute-Control plants is indistinguishable from the corresponding Null-Control plants (P=0.71, sodium-salt stress; P=0.29, aluminum-salt stress; Supplemental Material Results), indicating that plants receiving bacteria-free filtrate performed as if they had received a Null-Control treatment. Although microbiomes were selected to increase above-ground biomass of pre-

675 flowering plants (20-30 days old), selected microbiomes enhanced also seed production of older plants (68
676 days old).



677

678 **Figure 4. Specific and non-specific growth-enhancing effects of artificially selected microbiomes.** In
679 Generation 9, a 2x2 cross-fostering experiment tested whether microbiomes selected under sodium-salt
680 stress conferred greater salt tolerance to plants stressed with sodium-salt compared to plants stressed with
681 aluminum-salt; and conversely, whether microbiomes selected under aluminum-salt stress conferred greater
682 salt tolerance to plants stressed with aluminum-salt compared to plants stressed with sodium-salt. P-values
683 are shown for each comparison, and P-values are corrected using the false discovery rate for post-hoc
684 comparisons (Supplemental Material Results). **Findings:** The effect of microbiomes selected to confer
685 tolerance to aluminum-salt appears to be non-specific because these microbiomes confer equal tolerance to
686 plants stressed with either sodium-salt or aluminum-salt ($P>0.5$; two rightmost panels), whereas the effect
687 of bacterial microbiomes selected to confer tolerance to sodium-salt appears specific, because these sodium-
688 selected microbiomes confer less salt tolerance, or confer no salt tolerance, to plants under aluminum-salt
689 stress ($P<0.002$; two leftmost panels).