Differences in stability of seed-associated microbial assemblages in response to invasion by phytopathogenic microorganisms

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Seeds are involved in the vertical transmission of microorganisms from one plant generation to another and consequently act as reservoirs for the plant microbiota. However, little is known about the structure of seed-associated microbial assemblages and the regulators of assemblage structure. In this work, we have assessed the response of seed-associated microbial assemblages to invading phytopathogenic agents, the bacterial strain *Xanthomonas campestris* pv. *campestris* (*Xcc*) 8004 and the fungal strain *Alternaria brassicicola* Abra43. According to the indicators of bacterial (16S rRNA gene and *gyrB* sequences) and fungal (ITS1) diversity employed in this study, seed transmission of the bacterial strain *Xcc* 8004 did not changed the overall composition of resident microbial assemblages. In contrast seed transmission of Abra43 strongly modified the richness and structure of fungal assemblages without affecting bacterial assemblages. The sensitivity of seed-associated fungal assemblage to Abra43 is mostly related to changes in relative abundance of closely related fungal species that belong to the *Alternaria* genus. Variation in stability of the seed microbiota in response to *Xcc* and Abra43 invasions could be explained by differences in seed transmission pathways employed by these micro-organisms, which ultimately results in divergence in spatio-temporal colonization of the seed habitat.

Title : Differences in stability of seed-associated microbial assemblages in response to invasion by phytopathogenic microorganisms

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Abstract (196 words)

Seeds are involved in the vertical transmission of microorganisms from one plant generation to another and consequently act as reservoirs for the plant microbiota. However, little is known about the structure of seed-associated microbial assemblages and the regulators of assemblage structure. In this work, we have assessed the response of seed-associated microbial assemblages to invading phytopathogenic agents, the bacterial strain *Xanthomonas campestris* pv. *campestris* (*Xcc*) 8004 and the fungal strain *Alternaria brassicicola* Abra43. According to the indicators of bacterial (16S rRNA gene and *gyrB* sequences) and fungal (ITS1) diversity employed in this study, seed transmission of the bacterial strain *Xcc* 8004 did not changed the overall composition of resident microbial assemblages. In contrast seed transmission of Abra43 strongly modified the richness and structure of fungal assemblages without affecting bacterial assemblages. The sensitivity of seed-associated fungal assemblage to Abra43 is mostly related to changes in relative abundance of closely related fungal species that belong to the *Alternaria* genus. Variation in stability of the seed microbiota in response to *Xcc* and Abra43 invasions could be explained by differences in seed transmission pathways employed by these micro-organisms, which ultimately results in divergence in spatio-temporal colonization of the seed habitat.

42 **Introduction (887 words)**

43 Seeds are not only carriers of plants genetic information but are also involved in the
44 vertical transmission of microorganisms from one plant generation to another and consequently
45 act as reservoirs for the plant microbiota (Baker and Smith, 1966; Nelson, 2004). The activity of
46 seed-associated microbial assemblages is significant for plant growth and plant health since these
47 microbial assemblages may release seed dormancy through production of cytokinins (Goggin et
48 al., 2015) or limit the installation of microbial invader (Bacilio-Jimenez et al., 2001). Although
49 transmission of microorganisms from plant to seed is the primary source of inoculum for the
50 plant, relatively little is known about the structure of seed-associated microbial assemblages and
51 the regulators of assemblage structure (Johnston-Monje and Raizada, 2011; van Overbeek et al.,
52 2011; Lopez-Velasco et al., 2013; Links et al., 2014; Barret et al., 2015; Klaedtke et al., 2015).
53 Seeds acquire their microbiome by three majors' pathways: (*i*) internal transmission
54 through the vascular system, (*ii*) floral transmission by the stigma and (*iii*) external transmission
55 via contact of the seed with microorganisms present on fruits, flowers or residues (Maude, 1996).
56 According to the transmission pathway, seed-borne microorganisms can therefore be located on
57 different micro-habitats ranging from the testa to the embryo (Singh and Mathure, 2004; Dutta et
58 al., 2012; Tancos et al., 2013). While the internal transmission by the host xylem is restricted to
59 vascular pathogens or endophytic micro-organisms (Maude, 1996), many plant associated micro-
60 organisms are potentially transmitted to the seed by the floral pathway (Shade et al., 2013;
61 Aleklett et al., 2014). Indeed, the floral pathway allows the transmission of biocontrol
62 microorganisms (Spinelli et al., 2005) and phytopathogens (Darsonval et al., 2008; Darrasse et
63 al., 2010; Terrasson et al., 2015). Finally, the external pathway is probably the most permissive

64 way of microorganism transmission from plant to seed, although very little data are currently
65 available in the literature (Ngugi and Scherm, 2006).

66 Owing to the importance of seed transmission in emergence of diseases in new planting
67 areas, the processes involved in the transmission of microorganisms from plant to seed have been
68 mainly documented for phytopathogenic agents. The molecular determinants involved in
69 successful transmission of microorganisms from plant to seed have been notably studied in
70 bacteria related to the *Xanthomonas* genus (Darsonval et al., 2008; Darsonval et al., 2009;
71 Darrasse et al., 2010; Dutta et al., 2014) and fungi that belonged to *Alternaria brassicicola*
72 (Pochon et al., 2012; Pochon et al., 2013). Key molecular determinants like the bacterial Type III
73 Secretion System (Darsonval et al., 2008), bacterial adhesins (Darsonval et al., 2009), fungal
74 class III histidine-kinase or dehydrin like proteins (Pochon et al., 2013) have been shown to be
75 involved in seed transmission. In turn, seeds may respond to pathogen transmission through
76 activation of plant defenses and subsequent repression of seed maturation pathways (Terrasson et
77 al., 2015).

78 Although the host immune system is a decisive environmental filter to limit the
79 installation of an invader (Jones and Dangl, 2006), the host-associated microbial community may
80 also strongly prevent this invasion (Mendes et al., 2011). The resistance of microbial community
81 to invasion is linked to its level of diversity, since highly diverse microbial community are
82 usually less sensitive to invasion (Jousset et al., 2011; van Elsas et al., 2012) as a result of
83 enhanced competition for resources within species-rich community (Mallon et al., 2015a; Mallon
84 et al., 2015b; Wei et al., 2015). Because of its relative low microbial diversity (Lopez-Velasco et
85 al., 2013; Links et al., 2014; Barret et al., 2015; Klaedtke et al., 2015) compared to other plant
86 habitat such as the phyllosphere (Vorholt, 2012) or the rhizosphere (Hacquard et al.), mature

seed is an interesting experimental model to study biological disturbance. Moreover, mature seeds have low moisture content and are almost metabolically inactive (Dekkers et al., 2015), which suggest that associated micro-organisms are probably dormant and that the structure of microbial assemblages is in a stable state. Hence shift in assemblage structure are likely to reflect the outcome of the response of the microbial assemblage during seed transmission of phytopathogenic micro-organisms.

The aim of the present work was to analyze the impact of two microbial invaders, namely the bacterial strain *Xanthomonas campestris* pv. *campestris* (*Xcc*) 8004 and the fungal strain *A. brassicicola* (*Ab*) Abra43 on the structure of seed-associated microbial assemblages. We choose these two microbial invaders since they differ in their seed transmission pathways. Indeed *Xcc* is mostly transmitted from plant to seeds by the systemic and floral pathways (Cook et al., 1952; Van Der Wolf and Van Der Zouwen, 2010; van der Wolf et al., 2013), while *Ab* is transmitted by the external pathway and thus mostly restricted to the testa (Knox-Davies, 1979). Radish (*Raphanus sativus*) was used as an experimental system since this plant has a high seed multiplication ratio (1: 500 on averages) and a low microbial diversity compared to other Brassicaceae species (Barret et al., 2015). Profiling of seed-associated microbial assemblages was performed on mature seeds harvested from uninoculated plants or plants inoculated either with Abra43 or *Xcc* 8004 through sequencing of two bacterial taxonomic markers (16S rRNA gene and *gyrB*) and one fungal taxonomic marker (ITS1 region of the fungal internal transcribed spacer). This work revealed that the plant pathogenic fungal strain Abra43 had a significant impact on the fungal assemblages, while *Xcc* 8004 transmission did not impact the structure of seed microbiota.

Materials & Methods (1,654 words)

Site description, inoculation process and seed collection

Experiments were performed at the FNAMS experimental station (47°28'12.42"N, 0°23'44.30"W, Brain-sur-l'Authion, France) in 2013 and 2014 on 3 distinct plots (5 x 10 meters). Each plot was initially sown with *Raphanus sativus* var. Flamboyant on March 28th 2013. One plot (X2013) was spray-inoculated (approximately 100 $ml.m^{-2}$) at floral stage (June, 18th 2013) with *Xanthomonas campestris* pv. *campestris* (*Xcc*) strain 8004 (Qian et al., 2005). The spontaneous rifampicin-resistant bacterial strain *Xcc* 8004 was cultivated on Tryptic Soy Agar (TSA) 100% (17 $g.l^{-1}$ tryptone, 3 $g.l^{-1}$ soybean peptone, 2.5 $g.l^{-1}$ glucose, 5 $g.l^{-1}$ NaCl, 5 $g.l^{-1}$ K2HPO4, and 15 $g.l^{-1}$ agar) medium with rifampicin 50 $mg.l^{-1}$ for 2 days at 28°C. Bacterial colonies of *Xcc* 8004 were suspended in sterile deionized water at a final concentration of 1.10^7 CFU/ml. Another plot (A2013) was inoculated with *Alternaria brassicicola* strain Abra43 (Avenot et al., 2005) at the end of flowering (July 05th 2013) and at silique-formation stage (July 17th 2013) following protocol described earlier (Iacomi-Vasilescu et al 2008). The last plot (C2013) was not inoculated and subsequently used as a control plot.

A second experimentation was performed in 2014. Seeds harvested from C2013 were sown on April 2th, 2014 following the same experimental design as in 2013. The only difference between 2013 and 2014 experiments rely in *Xcc* 8004 inoculations. While in 2013 plants were inoculated once, two inoculations were performed in 2014 in order to increase the efficiency of *Xcc* 8004 transmission from plant to seed. Inoculation of *Xcc* 8004 were done at the beginning (June, 18th 2014) and at the end (June, 25th 2014) of flowering stage. Inoculation with *Ab* (June,

26th 2014 and July 7th 2014) was performed in one plot (A2014) using the same protocol as during the 2013 experimentation. The last plot (C2014) was left uninoculated.

At the mature seeds stage, seeds from plots C2013, A2013 and X2013 were harvested on September, 5th 2013, while seeds from plots C2014, A2014 and X2014 were collected on August, 22th 2014. Eighteen plants of each plot were collected and seeds were harvested manually for each individual plant. The remaining plants of each plot were harvested with a threshing machine leading to 6 seed lots (C2013, C2014, A2013, A2014, X2013 and X2014). Each of these seed lots was further divided into subsamples of 1,000 seeds (as assessed by 1,000 seeds weight).

Culture-based detection of phytopathogenic agents on seed samples

The transmission of *Xcc* 8004 from plants to seeds was initially evaluated through microbiological analysis of seed lots harvested with the threshing machine. Individual seeds were deposited in a 96 well-plate and soaked in 200 µL of phosphate buffer saline (PBS) supplemented with 0.05% (v/v) of Tween® 20 during 2 hours and 30 minutes at room temperature under constant agitation (140 rpm). Then 10 µl of each well was spread on TSA 10% medium supplemented with rifampicin (50 mg/l). Plates were incubated at 28°C for 2 days and the presence of *Xcc* 8004 was evaluated for each individual seed. Contamination rate of each seed lot is a mean of six biological replicates, each containing 96 seeds. The transmission of *Alternaria brassicicola* strain Abra43 (*Ab*) from plant to seeds was assessed by standard plating technique (Iacomi-Vasilescu et al., 2008). Briefly, 10 individual seeds were deposited on potato-dextrose agar (4 $g.l^{-1}$ potato extract, 20 $g.l^{-1}$ dextrose, 15 $g.l^{-1}$ agar) and plates were incubated at 25°C for 5 days. *Alternaria brassicicola* isolates were further purified by monospore isolation.

Contamination rate of each seed lot is a mean of 6 biological replicates, each biological replicate containing 100 seeds (10 plates per seed lot).

DNA extraction

DNA extraction were performed according to standard procedures recommended by the International Seed Testing Association (ISTA, https://www.seedtest.org/en/home.html). Briefly, seeds harvested manually on each plant or 1,000 seeds subsamples harvested mechanically were transferred in sterile tubes containing 25 mL of PBS supplemented with 0.05% (*v/v)* of Tween® 20. Samples were incubated during 2 hours and 30 minutes at room temperature under constant agitation (140 rpm). Suspensions were centrifuged (6000 x *g*, 10 min, 4°C) and pellets were resuspended in approximately 2 ml of supernatant and transferred in microtubes. Total DNA was extracted with the Power Soil DNA Kit (MoBio Laboratories) from 148 seed suspensions following procedure described earlier (Barret et al., 2015).

Molecular detection of phytopathogenic agents on seed samples

Seed transmission of phytopathogenic agents was also monitored by quantitative PCR. The number of copy of the predicted gene XC_1533 encoding a hypothetical protein was used as an estimator of the number of Xcc 8004 cells per seed samples (Rijlaarsdam et al., 2004). A portion of this gene was amplified with the primer set Zup4F/Zup4R (**Table S6**). Data normalization between seed samples was performed with a portion of the 16S rRNA gene, using the primers 926F/1062R (**Table S1**). All reactions were performed in 25 µl qPCR reaction using 12.5 µl of SYBR Green Master Mix (MESA BLUE qPCR MasterMix Plus for SYBR Assay; Eurogentec, Cologne, Germany), 2 µl of DNA (10 $ng.\mu l^{-1}$) and 0.5 µL of each primer (10 µM). Amplification conditions were 5 min at 95°C, followed by 40 two-step cycles of 95°C (15s) and 60°C (60s).

177 The number of copies of the gene AbDhn1 encoding a dehydrin-like protein was used as
178 an estimator of the number of Abra43 cells (Pochon et al., 2013) using the primer set
179 AbraDHN1-Tq-F/AbraDHN1-Tq-R and a TaqMan MGB probe (**Table S1**). Reactions were
180 conducted using StepOnePlus™ qPCR system (Applied Biosystems). qPCR reaction were
181 performed in 20 µl using 10 µl of Master Mix, 2 µl of TaqMan probe (250 nM), 2 µl of each
182 primer (0.3 µM and 0.9 µM for the forward and the reverse primer respectively) and 2 µl of
183 DNA (10 ng.µl^{-1}). Amplification conditions were 10 min at 95°C, followed by 40 two-step
184 cycles of 95°C (15s) and 60°C (60s). Data normalization between seed samples was performed
185 with a portion of the β-actin gene, using the primers ACT 512-F/ACT 783-R (**Table S6**) and
186 amplification conditions described previously (Carbone and Kohn, 1999).
187
188 **Assessment of microbial diversity**
189 PCR amplification was conducted on 148 DNA samples and 2 artificial microbial community
190 samples containing a mixture of 15 DNA extracted from different bacterial strains (Barret et al
191 2015). Amplification were performed with the primer sets 515F/806R, gyrB_aF64/gyrB_aR353
192 and ITS1F/ITS2 (**Table S1**) following procedures described earlier (Barret et al., 2015).
193 Amplicons libraries were mixed with 7.5 % PhiX control according to Illumina's protocols. A
194 total of four sequencing runs were performed for this study with MiSeq Reagent Kits v2 (500
195 cycles).
196 Sequence analyses were performed with Mothur v1.31.2 (Schloss et al., 2009) using
197 standard operating procedure (Kozich et al., 2013) described earlier (Barret et al., 2015). Briefly,
198 16S rRNA gene and *gyrB* sequences were aligned against the 16S rRNA gene SILVA alignment
199 and a *gyrB* reference alignment, respectively. Chimeric sequences were detected with UCHIME

(Edgar et al., 2011) and subsequently removed from the dataset. Moreover, *gyrB* sequences containing stop codon were discarded. Taxonomic affiliation of 16S rRNA gene and *gyrB* sequences was performed with a Bayesian classifier (Wang et al., 2007) (80% bootstrap confidence score) against the 16S rRNA gene training set (v9) of the Ribosomal Database Project (Cole et al., 2009) or against an in-house *gyrB* database created with sequences retrieved from the IMG database (Markowitz et al., 2012; Barret et al., 2015). Unclassified sequences or sequences belonging to Eukaryota, Archaea, chloroplasts or mitochondria were discarded. Sequences were divided into groups according to their taxonomic rank (level of order) and then assigned to operational taxonomic units (OTUs) at 97% identity cutoff for 16S rRNA gene and 98% identity for *gyrB* sequences. The variable ITS1 regions of ITS sequences were extracted with the Perl-based software ITSx (Bengtsson-Palme et al., 2013). Then sequences were clustered at a 97% identity cut-off using Uclust (Edgar, 2010) and taxonomic affiliation was performed with a Bayesian classifier (Wang et al., 2007) (80% bootstrap confidence score) against the UNITE database (Abarenkov et al., 2010). To improve the resolution of the taxonomic classification of ITS1 sequences, we performed a reciprocal blast analysis at a minimum cut-off of 97% with representatives OTUs sequences and available ITS1 sequences of *Alternaria* type strains (Woudenberg et al., 2013). In order to enhance the reproducibility of community profiles, abundant OTUs (aOTU) representing at least 0.1% of the library size were used for microbial community analyses (Barret et al., 2015).

In order to avoid biases introduced by unequal sampling, total counts were divided by library size of each sample sequenced (McMurdie and Holmes, 2014). Both alpha and beta diversity indexes were calculated with Mothur (Schloss et al., 2009). Richness was defined as the number of different OTUs and aOTUs per sample. Hierarchical clustering of different seed

223 samples was performed using an average linkage method based on Bray-Curtis dissimilarity
224 index (Bray and Curtis, 1957) and on unweighted UniFrac distances (Lozupone and Knight,
225 2005). Analysis of similarity (ANOSIM) was used to assess the effects of the different
226 conditions on the microbial community structure. Moreover, canonical analysis of principal
227 coordinates (CAP) was conducted to measure the relative influence of (*i*) the phytopathogenic
228 agent, (*ii*) the harvesting year and (*iii*) the harvesting method on microbial β-diversity. CAP
229 analyses were performed with the function capscale of vegan.

230 Correlation between aOTUs were calculated with Sparse Correlation for Compositional
231 data algorithm (Friedman and Alm, 2012) implemented in Mothur. Statistical significance of the
232 inferred correlations was assessed with a bootstrap procedure (100 replications). Only
233 correlations with pseudo *p*-value ≤ 0.001 were represented in the network using the R package
234 qgraph (Epskamp et al., 2012). Changes in relative abundance of aOTUs between the different
235 experimental conditions (C, X and A) were assessed with LEfSE (Segata et al., 2011). aOTUs
236 were defined as significantly enriched or depleted in one treatment at a *P* value ≤ 0.05 and a
237 LDA score > 2.

238 All sequences have been deposited in the ENA database under the accession number
239 PRJEB9588.

240

Results (1,909 words)

The impact of pathogen transmission on the structure of seed-associated microbial assemblages was assessed on radish seed lots harvested in 2013 and 2014 from plots inoculated with the bacterial strain *Xanthomonas campestris* pv. *campestris* 8004 (X2013 and X2014 plots), with the fungal strain *Alternaria brassicicola* Abra43 (A2013 and A2014 plot) and from uninoculated plants (C2013 and C2014 for control plots).

Efficient transmission of phytopathogenic microorganisms to seeds

The seed transmission of *Xcc* 8004 and *Ab* Abra43 was first evaluated by standard microbiological approaches on seed samples collected in 2013 and 2014. According to these microbiological analyses, *Ab* was not detected on seeds harvested from control plots (C2013 and C2014), while a residual *Xcc* contamination of 0.69 % and 0.13 % was observed in seeds from C2013 and C2014 samples, respectively (**Fig. 1A** and **Fig. 1B**). However, the incidence of *Xcc* increased significantly in X2013 and X2014 samples ($P< 0.01$, as assessed by 2-sample test for equality of proportions) with 6% and 26% of seeds contaminated, respectively (**Fig. 1A**). *Ab* was not detected in control samples, nevertheless a significant increase of *Ab* incidence ($P< 0.01$) was observed in A2013 and A2014 samples with 54% and 36% of seed contaminated (**Fig. 1B**), respectively.

To confirm these results, qPCR experiments were performed on DNA extracted from seed samples with primers and probes targeting XC_1533, a single-copy gene of *Xcc* encoding a hypothetical protein (Rijlaarsdam et al., 2004), and *AbDhn1*, a single-copy gene of *Ab* encoding a dehydrin-like protein (Pochon et al., 2013). A significant increase ($P< 0.01$, as assessed by ANOVA with post hoc Tukey's HSD test) in copy number of XC_1533 was observed on X2013

(1.10^5 copies) and X2014 (4.10^6 copies) samples in comparison to control samples (**Fig. 1C**). Similarly, the number of copy of *AbDhn1* also increased ($P< 0.01$) in A2013 (1.5.10^{10}) and A2014 (4.5.10^{10}) (**Fig. 1D**). These differences were not due to variation in DNA amounts between seed samples since the copy numbers of 16S rRNA and β-actin genes were not significantly different between seed samples (**Fig. S1**). Altogether microbiological and qPCR analyses highlighted an effective transmission of *Xcc* 8004 and *Ab* Abra43 from plant to seed.

Seed-associated fungal assemblages are impacted by *Ab* Abra43 transmission

The structure of 148 microbial assemblages associated to seeds harvested in C2013, C2014, A2013, A2014, X2013 and X2014 was assessed through amplification and subsequent sequencing of two bacterial molecular markers (16S rRNA gene and *gyrB*) and one fungal molecular marker (ITS1). A total of 7,870,622 (16S rRNA gene), 24,355,191 (*gyrB*) and 8,799,598 (ITS1) paired-end reads were obtained (**Table S2**). Reads were assembled in quality sequences and grouped into operational taxonomic units (OTUs) at ≥ 97% sequence identity for 16S rRNA gene and ITS1 sequences and ≥ 98% sequence identity for *gyrB*. (**Table S2**). To increase the reproductibility of OTU detection between samples, OTUs with a relative abundance ≥0.1% of the library size were defined as abundant OTUs (Barret et al., 2015). However, this threshold remove rare OTUs, which contributes to a large amount of diversity observed within microbial assemblages (Shade et al., 2014). Therefore, subsequent analyses were performed (*i*) on every OTUs including abundant and rare OTUs and (*ii*) on abundant OTUs (aOTUs) only. Since seed samples used in this study have been either harvested manually on individual plants or mechanically with a threshing machine (see experimental procedures for further informations), the influence of the harvesting method on the structure of seed-associated

287 microbial assemblages was first investigated. According to ANOSIM tests, the harvesting
288 method did not impact the structure of microbial assemblages (**Table 1**).

289 The impact of pathogen transmission was evaluated on microbial richness using OTU and
290 aOTU counts as proxies for species richness. Overall, the number of bacterial OTU (**Fig. S2A**
291 and **Fig. S2B**) and aOTU (**Fig. 2A** and **Fig. 2B**) was constant between control samples and seed
292 harvested from A2013, A2014, X2013 and X2014. Therefore, the presence of *Xcc* 8004 and *Ab*
293 Abra43 within the seed microbiota does not seem to alter bacterial richness. While fungal
294 richness was also not affected by seed transmission of *Xcc* 8004, the number of fungal OTU and
295 aOTU significantly (P <0.01, as assessed by ANOVA with post hoc Tukey's HSD test)
296 decreased for A2013 and A2014 (**Fig. 2C** and **Fig. S2C**). Hence *Ab* Abra43 seems to reduce the
297 number of resident fungal taxa associated to radish seeds.

298 We next measured the effect of *Xcc* and *Ab* transmission on microbial diversity.
299 According to inverse Simpson diversity index, bacterial α-diversity was neither affected by *Xcc*
300 8004 nor *Ab* Abra43 transmission (**Fig. 2D**, **Fig. 2E**, **Fig. S2D** and **Fig. S2E**). Changes in
301 resident bacterial assemblages between samples was further estimated by Bray-Curtis
302 dissimilarity measure (Bray and Curtis, 1957) and unweighted UniFrac distance (Lozupone and
303 Knight, 2005) using OTU and aOTU counts obtained with 16S rRNA gene and *gyrB* sequences.
304 According to hierarchical clustering and ANOSIM tests, neither *Xcc* 8004 nor *Ab* Abra43
305 impacted the structure of seed-associated bacterial assemblages (**Fig. S3**, **Fig. S4** and **Table 1**).

306 Regarding seed-associated fungal assemblages, no significant difference in α-diversity
307 was observed between control seed samples and samples contaminated with *Xcc* (**Fig. 2F** and
308 **Fig. S2F**). However, a significant reduction of inverse Simpson diversity index was observed
309 following invasion of seed-associated fungal assemblages by *Ab* (**Fig. 2F** and **Fig. S2F**). To gain

more insight on the influence of *Ab* on seed-associated fungal assemblages, β-diversity was estimated with Bray-Curtis dissimilarity measure. A significant disturbance ($p<0.001$) of seed-associated fungal assemblages was observed for samples harvested in A2013 and A2014 (**Fig. 3** and **Table 1**). Indeed all these seed samples grouped together, which indicates that transmission of *Ab* Abra43 from plant to seed has a profound influence on the structure of seed-associated fungal assemblages (**Fig. 3**). According to canonical analysis of principal coordinates (CAP), the seed transmission of *Ab* was explaining 63% ($p<0.001$) of the variation in fungal diversity across seed samples.

Shift in relative abundance of microbial taxa following seed transmission of *Ab* Abra43

The taxonomic composition of seed-associated microbial assemblages was investigated in samples harvested in C2013 and C2014. According to both 16S rRNA gene and *gyrB* sequences, bacterial aOTUs were mostly affiliated to Enterobacteriales and Pseudomonadales (**Fig. S5**), which confirms that taxonomic classification performed with these two molecular markers give similar results at high taxonomic rank (e.g. order level). Distribution of bacterial aOTU was then investigated across seed samples. Only 3 16S rRNA aOTUs affiliated to *Pantoea* (Otu00001) and *Pseudomonas* (Otu00002 and Otu00003) were shared across all seed samples (**Fig. S3**). Three *gyrB* aOTUs corresponding to *Pantoea agglomerans* (Otu00001), *Pseudomonas viridiflava* (Otu00002) and *Erwinia tasmaniensis* (Otu00003) were also conserved between all samples (**Fig. S4**). These aOTUs were highly-abundant in all seed samples with an average relative abundance of 58% (*P. agglomerans*), 12% (*P. viridiflava*) and 4% (*E. tasmaniensis*) of all *gyrB* sequences. While we did not identify bacterial aOTUs specifically associated to C, A and X samples, significant changes (p-value ≤ 0.05 and LDA score ≥ 2) in relative abundance of

bacterial aOTUs were observed with LEfSE (Segata et al., 2011). Unsurprisingly, the relative abundance of aOTUs affiliated to *Xanthomonas* (Otu0004 - 16S rRNA gene sequences) and *Xanthomonas campestris* (Otu00039 - *gyrB* sequences) were both increased in seed samples harvested from X2013 and X2014 (**Table S3** and **Table S4**). The increase in relative abundance of these *Xanthomonas*-related aOTUs was associated with changes in relative abundance of bacterial aOTUs belonging to the Pseudomonodaceae and Enterobacteriaceae (**Table S3** and **Table S4**) and of fungal aOTUs mainly related to *Alternaria* (**Table S5**).

Regarding fungal assemblage composition, the seed microbiota of C2013 and C2014 samples was mainly composed of Pleosporales (**Fig. S5**). A significant shift ($p < 0.01$) in relative abundance of this fungal order was observed in A2013 and A2014 samples (**Fig. S5**). Since *Ab* belonged to the Pleosporales, this increase is probably due to the seed transmission of this pathogenic agent. At the aOTU-level 8 fungal entities were conserved in all seed samples (**Fig. 3**), five of them were affiliated to Pleosporales, two to Capnodiales and one to Basidiomycota. According to LEfSE analysis, 7 aOTUs were enriched in A2013 and 2014 samples (**Fig. 3** and **Table S5**). These entities belonged to *Alternaria* and three of them are affiliated to *Alternaria* sect. *brassicicola* (OTU0443, OTU0446 and OTU0937). However, closer examination of the representative sequences of these aOTUs revealed that Abra43 belonged to OTU0446 and is the dominant phylotype of this group (96% of all reads included in OTU0446). Seed transmission of Abra43 also results in decrease in relative abundance of 61 fungal aOTUs that belonged to various fungal genus including *Alternaria*, *Cladosporium* or *Fusarium* (**Table S5**). In addition 7 (16S rRNA gene sequences) and 14 (*gyrB* sequences) bacterial aOTUs were also impacted by Abra43 transmission (**Table S3** and **Table S4**).

Correlation between microbial taxa within the seed microbiota

In order to predict microbial interactions within seed-associated assemblages, we explored positive and negative associations between entities of these assemblages by generating correlations networks with SparCC (Friedman and Alm, 2012). Considering only inferred correlations with pseudo *p*-values ≤0.001, we identified 15 and 100 nodes in control samples with 16S and *gyrB* aOTUs, respectively (**Fig. 4, Fig. S6** and **Table S6**). These nodes were sharing a total of 52 edges with 16S aOTUs and 206 edges with *gyrB* aOTUs. The other bacterial correlations networks generated with samples from A and X plots were not different from the network inferred with samples from control plots (**Fig. 4, Fig. S6** and **Table S6**). Indeed all bacterial networks were split into multiples small modules with low connectivity between nodes. Moreover these inferred correlation networks were characterized by nodes having a maximal degree of 4 to 7 edges for 16S OTUs and 4 to 6 connections for *gyrB* aOTUs.

In comparison to bacterial networks, correlations networks inferred from seed-associated fungal assemblages were composed of more edges and less modules (**Fig. 4** and **Table S5**), suggesting more interactions between entities of these assemblages. Seed transmission of *Xcc* 8004 did not impact the overall structure of the inferred fungal network, since the number of nodes, edges and median number of connectivity were constant between samples from C and X plots. Moreover, the highest degree nodes of both networks were related to fungal aOTUs conserved in each seed samples such as OTU0449 (*Alternaria brassicae*) for C and OTU0536 (*Alternaria* sect. *infectoria*) for X plots. On the contrary, *Ab* Abra43 strongly impacted the topology of the network with a decrease of nodes, edges and median number of connectivity (**Fig. 4** and **Table S6**). Although OTU0449 and OTU0536 are still part of the fungal network in

samples from A plots, these entities are not hubs anymore and shared 2 and 5 connections with other nodes, respectively.

Based on *gyrB* and 16S rRNA gene datasets, we were not able to detect inferred correlations between the bacterial aOTU affiliated to *Xcc* and other entities of seed-associated bacterial assemblages. Analysis of inferred correlation between *Ab* (OTU0446) and other fungal aOTUs revealed positive correlations with 7 entities that belong exclusively to *Alternaria*. Four of these aOTUs were already highlighted by hierarchical clustering (**Fig. 3**). In addition, 12 negative correlations between OTU0446 and other fungal entities were observed. Some of these fungal aOTUs were affiliated to *Alternaria* but also to other fungal orders such as Filobasidiales and Sporidiobolales. Interestingly the highest degree node of the correlation network inferred in samples harvested from A plot (OTU0309) was negatively associated to *Ab*, suggesting that seed transmission of this phytopathogenic fungus severely impacted resident fungal community.

Discussion (833 words)

The aim of the present study was to assess the influence of two microbial invaders, namely *Xanthomonas campestris* pv. *campestris* (*Xcc*) 8004 and *A. brassicicola* (*Ab*) Abra43, on the structure of resident microbial assemblages associated with seeds. According to our indicators of bacterial (16S rRNA gene and *gyrB* sequences) and fungal (ITS1) diversity, seed transmission of the bacterial strain *Xcc* 8004 did not impact the overall composition of seed-associated microbial assemblage. In contrast, transmission of Abra43 significantly changed the structure of resident fungal assemblages without altering bacterial assemblages' composition.

Variation in response of resident microbial assemblages to invasion of *Xcc* 8004 and Abra43 could be explained by the distinct transmission pathways employed by these two micro-organisms to invade seeds, which ultimately result in differences of spatio-temporal distribution of these strains within the seed habitat. *Xcc* has been reported to invade seeds via the vascular tissue of the mother plant (Cook et al., 1952) or by flower infection during pollination (Van Der Wolf and Van Der Zouwen, 2010). Therefore, successful seed transmission of *Xcc* is probably strongly dependent of local resources available in the plant xylem or on flower surface. Conversely, *Ab* invades the seed via colonization of the pods and subsequent migration through the funicles (Singh and Mathure, 2004) and thus interacted mostly with microbial taxa associated to pods. As a consequence of these different routes of infection, *Xcc* is mostly located in the endosperm or inner integuments (Maude, 1996), while *Ab* is frequently isolated from the hilum of the seed coat (Knox-Davies, 1979). Therefore the contrasted response of seed-associated microbial assemblages to invasion by *Xcc* or *Ab* could be due to differences in microbial interactions occurring within these distinct micro-habitats. Differences in seed transmission pathways between *Xcc* and *Ab* also results in distinct timing of seed colonization, *Xcc* being

414 associated with earlier seed development stage. Recently, it has been hypothesized that assembly
415 history may determine the structure of seed-associated bacterial assemblages (Aleklett and Hart,
416 2013; Klaedtke et al., 2015) in a similar manner than other plant-related habitat such as the
417 phyllosphere (Maignien et al., 2014). Therefore, the resistance of seed-associated bacterial
418 assemblage to *Xcc* 8004 and Abra43 invasions could be due to prior colonization of the seed by
419 pioneer bacterial entities, which maintain community structure (Shade et al., 2012). Although
420 this hypothesis has to be tested experimentally through temporal survey of microbial
421 assemblages during the different seed development stages, it is tempting to speculate that these
422 pioneer species may be related to bacterial taxa such as *Erwinia*, *Pseudomonas* or *Pantoea.*
423 Indeed these taxa are highly abundant within the seeds samples collected in this study and have
424 been frequently observed in flower (Aleklett et al., 2014) and seeds (Johnston-Monje and
425 Raizada, 2011; Links et al., 2014; Barret et al., 2015; Klaedtke et al., 2015) of several plant
426 species.

427 Alternatively, we cannot rule out the possibility that the observed stability of microbial
428 assemblage in response to *Xcc* 8004 transmission is partly due to the low abundance of *Xcc*
429 detected within seed samples contaminated with this bacterial strain. Although the incidence of
430 transmission of *Xcc* on radish seeds is in accordance with previous studies (Cook et al., 1952;
431 Van Der Wolf and Van Der Zouwen, 2010; van der Wolf et al., 2013) and reflected the natural
432 seed transmission observed with other bacterial pathogens (Darrasse et al., 2007), the high
433 prevalence of seeds not contaminated with *Xcc* in X2013 (94%) and X2014 (72%) samples
434 together with a low *Xcc* population sizes may result in a high background signal that is likely to
435 mask changes occurring within microbial assemblages. In comparison the high incidence and

436 abundance of *Ab* within A2013 and A2014 seed samples allow detection of changes in microbial
437 assemblages' structure.

438 In contrast to results obtained with *Xcc*, the seed transmission of Abra43 significantly
439 impacted the structure of fungal assemblages. Perturbation of fungal assemblage following *Ab*
440 transmission is probably explained by competition between *Ab* and other functional equivalent
441 species for resources and spaces (Burke et al., 2011). Indeed the transmission of *Ab* from plant to
442 seed is correlated with a decrease in relative abundance of closely related entities that belong to
443 *Alternaria brassicae*, *Alternaria.* sect. *infectoria* and *Alternaria.* sect. *alternate* (Woudenberg et
444 al., 2013). As these fungal aOTUs represented hubs of inferred fungal correlation network in
445 native condition, the potential competition of these entities with *Ab* result in a drastic shift in the
446 structure of seed-associated fungal assemblage. Among the observed changes, we observed
447 multiple co-occurrences between *Ab* and other fungal entities related to *Alternaria* sect.
448 *brassicicola*. Whether these entities either interact positively or are selected in similar ways by
449 the environment remained to be determined.

450 The results of this work provide a first glimpse into the response of the seed microbiota
451 following seed transmission of two phytopathogenic microorganisms. Future metagenomics
452 analysis of seed-associated microbial assemblages will be useful to assess the relationship
453 between assemblage structure and function. This research might lead to the development of
454 biocontrol strategies based on the potential of seed-associated microbial community.

455

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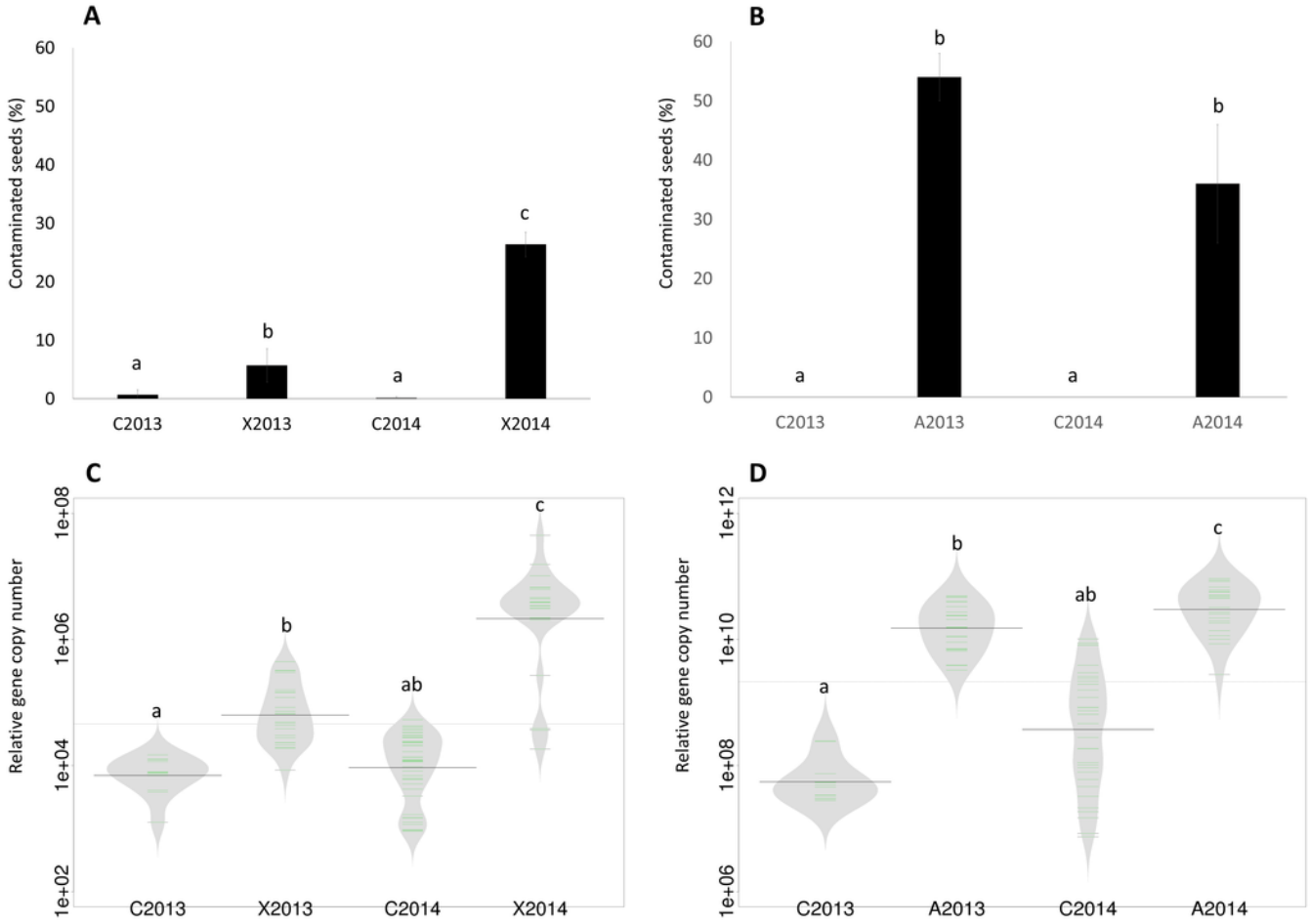
1

Figure 1: Assessment of seed contamination by *X. campestris* pv. *campestris* 8004 and *A. brassicicola* Abra43

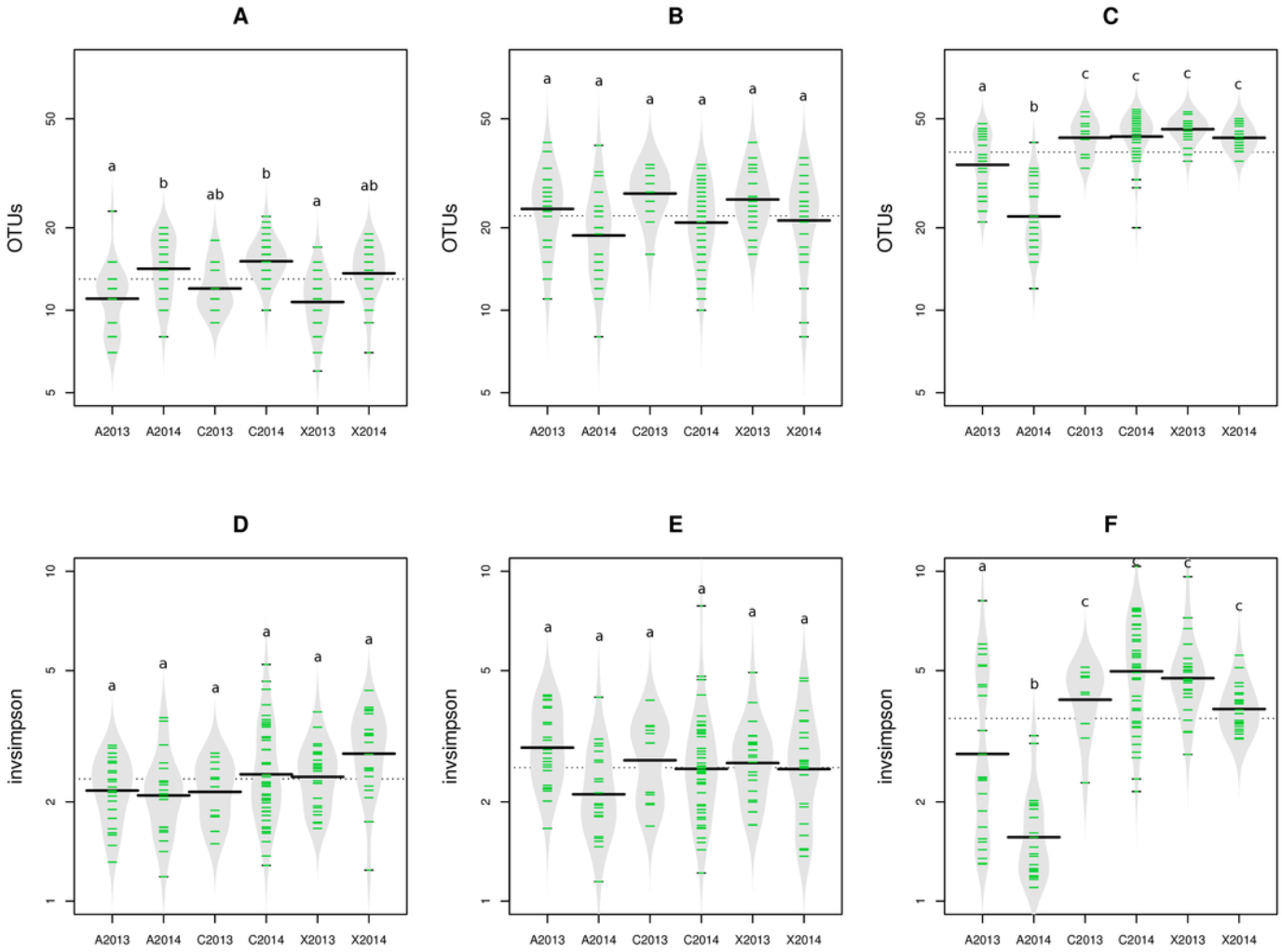
The contamination rate of seed samples by *X. campestris* pv. *campestris* 8004 (**A**) and *A. brassicicola* Abra43 (**B**) was assessed through microbiological analysis of 96 and 100 individual seeds, respectively. Contamination rates are the mean of 6 independent biological replicates performed for each experimental year. Quantitative detection of *Xcc* (**C**) was performed on seed samples harvested from uninoculated plants (C2013 and C2014) and plants inoculated with *Xcc* (X2013 and X2014) through qPCR with primers targeting XC_1533. Quantitative detection of *Ab* (**D**) was performed on seed samples harvested from uninoculated plants (C2013 and C2014) and plants inoculated with *Ab* (A2013 and A2014) through qPCR with primers and probe targeting *AbDhn1*. The green lines represent the number of each target gene in the different samples, while bold dark black lines represent the median. The grey area indicates the density of distribution. Differences in contamination rate and relative abundance were considered significant at a p-value ≤ 0.01 (as assessed by 2-sample test for equality of proportions and ANOVA with post hoc Tukey's HSD test, respectively).


A
Contaminated seeds (%)
60
50
40
30
20
10
0
a
b
a
c
C2013
X2013
C2014
X2014
B
Contaminated seeds (%)
60
50
40
30
20
10
0
a
b
a
b
C2013
A2013
C2014
A2014
C
Relative gene copy number
1e+08
1e+06
1e+04
1e+02
a
b
ab
c
C2013
X2013
C2014
X2014
D
Relative gene copy number
1e+12
1e+10
1e+08
1e+06
a
b
ab
c
C2013
A2013
C2014
A2014

2

Figure 2: Richness and diversity of seed samples observed with abundant OTUs

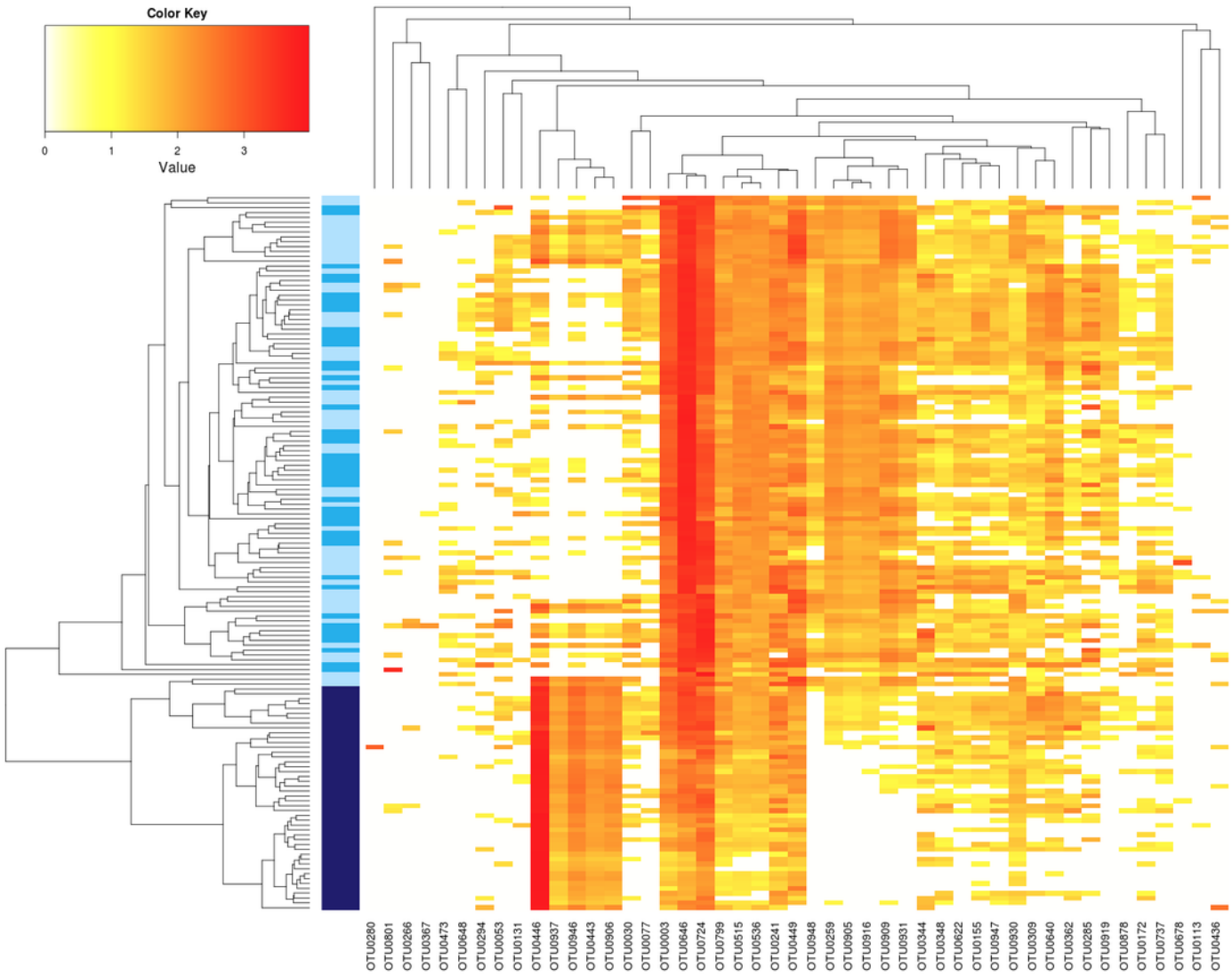
Microbial richness (A, B and C) and diversity (D, E and F) were estimated with abundant OTUs obtained with 16S rRNA gene (A and D), *gyrB* (B and E) and ITS1 sequences (C and F). Richness and diversity associated to uncontaminated seeds (C2013 and C2014), seeds contaminated with *Xcc* (X2013 and X2014) and seeds contaminated with *Ab* (A2013 and A2014) were compared. Each sample is represented by a green line, while black line represents the median. The grey area represents the density of distribution. Letters a, b and c denote significant changes between conditions considered at a *P*-value ≤ 0.01 (as assessed by ANOVA with post hoc Tukey's HSD test).


A
B
C
D
E
F
OTUs
invsimpson
A2013
A2014
C2013
C2014
X2013
X2014

3

Figure 3: Influence of *Ab* on the structure of seed-associated fungal assemblages

Hierarchical clustering of seed samples (*y* axis) is based on Bray-Curtis dissimilarity measure. The type of samples is represented by gradual color changes: light blue for controls, medium blue for seeds contaminated with *Xcc* and dark blue for seeds contaminated with *Ab*. Only abundant OTUs (threshold of 1% in relative abundance) are represented in the heatmap. These aOTUs are clustered by their co-occurrence patterns (*x* axis). According to analysis of similarity, a significant clustering of *Ab* seed samples was observed ($p < 0.001$).



4

Figure 4: Inferred correlations between aOTUs

Correlation networks between bacterial taxa are based on *gyrB* sequences obtained in uncontaminated seeds (A), seeds contaminated with *Xcc* (B), and contaminated with *Ab* (C). Correlation networks between fungal taxa are based on ITS1 sequences obtained in uncontaminated seeds (D), seeds contaminated with *Xcc* (E), and contaminated with *Ab* (F). Correlations between aOTUs were calculated with the Sparse Correlations for Compositional data algorithm. Each node represents an aOTUs, which is colored according to its taxonomic affiliation (family-level). Edges represent correlations between the nodes they connect with blue and orange colors indicating negative and positive inferred correlation, respectively. Only correlations with pseudo *p*-value ≤ 0.001 were represented in the network using the R package qgraph.

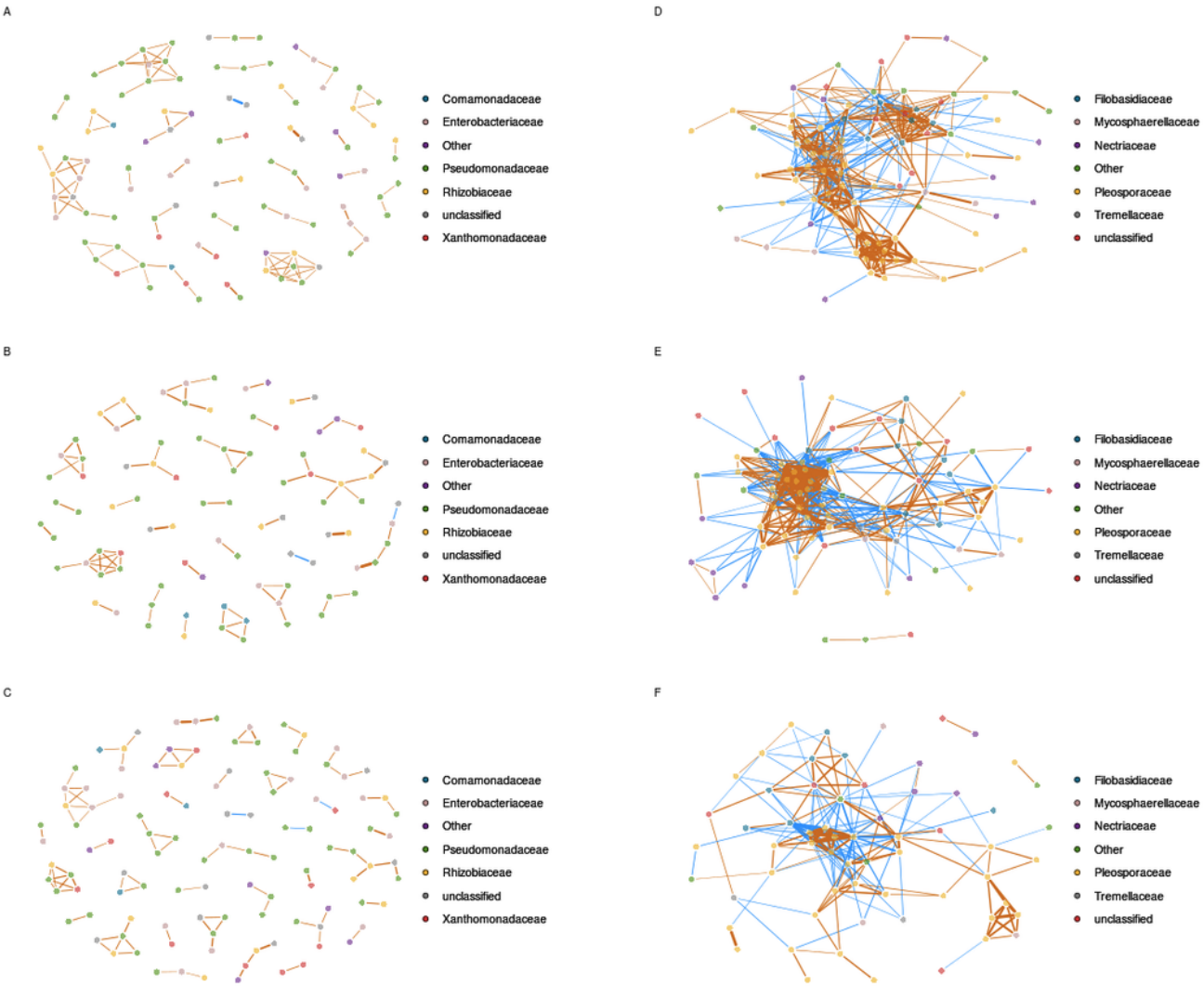

A
Comamonadaceae
Enterobacteriaceae
Other
Pseudomonadaceae
Rhizobiaceae
unclassified
Xanthomonadaceae
B
Comamonadaceae
Enterobacteriaceae
Other
Pseudomonadaceae
Rhizobiaceae
unclassified
Xanthomonadaceae
C
Comamonadaceae
Enterobacteriaceae
Other
Pseudomonadaceae
Rhizobiaceae
unclassified
Xanthomonadaceae
D
Filobasidiaceae
Mycosphaerellaceae
Nectriaceae
Other
Pleosporaceae
Tremellaceae
unclassified
E
Filobasidiaceae
Mycosphaerellaceae
Nectriaceae
Other
Pleosporaceae
Tremellaceae
unclassified
F
Filobasidiaceae
Mycosphaerellaceae
Nectriaceae
Other
Pleosporaceae
Tremellaceae
unclassified

Table 1(on next page)

Table 1: Analysis of similarity of seed-associated microbial assemblages

Analysis of similarity (ANOSIM) was used to assess the robustness of the hierarchical clustering analyses (Bray-Curtis dissimilarity measure and unweighted Unifrac distance). P-values are displayed in each column. Only *P*-values highlighted in bold are considered as significant

1

Marker	diversity index	OTU	A13vsC13	X13vsC13	A14vsC14	X14vsC14	Manual vs Mechanical
16S rRNA gene	Bray-Curtis	OTUs	0.693	0.249	0.932	0.045	0.991
		aOTUs	0.733	0.223	0.918	0.058	0.989
	Unifrac unweighted	OTUs	0.698	0.298	0.032	0.029	0.976
		aOTUs	0.737	0.407	0.044	0.018	0.962
gyrB	Bray-Curtis	OTUs	0.257	0.208	0.098	0.105	0.999
		aOTUs	0.156	0.138	0.041	0.028	0.998
	Unifrac unweighted	OTUs	0.241	0.520	0.134	0.804	0.999
		aOTUs	0.203	0.467	0.026	0.111	0.957
ITS1	Bray-Curtis	OTUs	**<0.001**	0.763	**<0.001**	0.504	0.999
		aOTUs	**<0.001**	0.777	**<0.001**	0.406	0.999

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