1   Seed sourcing for climate-resilient grasslands: the role of seed source diversity during early

2   restoration establishment

3   Running Head: Seed source diversity in restoration establishment

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13   experiments; JL analyzed the data; JL, MA, JH wrote and edited the manuscript.

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17   ABSTRACT

18   Restoration often advocates for the use of local seed in restoration, however increasingly

19   new strategies have been proposed to incorporate diverse sources to maintain evolutionary

20   potential within seed mixes. Increasing seed sources per species within a seed mix should

21   increase genetic variation, however, few empirical studies have evaluated how seed source

22   diversity impacts plant community composition following restoration. Thus, the goal of this

23   research was to compare the use of single or multi-source seed mix treatments to plant

24   community diversity following restoration. Using 14 species commonly applied in grassland

25   restoration, we examined plant community diversity following restoration comparing seed mixes

26   with either one or five sources per species across two restoration sites in Minnesota and South

27   Dakota, United States. Following seeding, species establishment and abundance were recorded to

28   calculate plant diversity for each seed mix treatment. There were no major effects of seed mix

29   treatment on community emergence and diversity observed, with the majority of plant

30   establishment reflecting non-seeded species. However, site-specific differences were observed.

31   Heterogeneous land-use history associated with the Minnesota site likely contributed to

32   differences across the restoration treatments. In contrast, community diversity at the South

33   Dakota site was homogeneous across seed mix treatments with changes in plant community

34   influenced solely by early season species establishment. This suggests land-use history

35   irrespective of seed mix treatment influences establishment and persistence, particularly in the

36   first year following restoration. Future monitoring across seasons will be needed to evaluate if

37   community diversity changes in response to seed mix treatment.

38

39   KEYWORDS: Grassland restoration, plant diversity, emergence, seed mix, species richness,

40   genetic variation

41   INTRODUCTION

42   One of the major aims of ecological restoration is to restore or re-establish functional

43   plant species diversity to ensure key ecosystem services are maintained (Barr et al. 2017;

44   Montoya et al. 2012). To ensure ecosystem health and the maintenance of productive plant

45   communities, this includes creating diverse seed mixes for application in restoration (Tilman et

46   al. 1996, 1997, 2001; Brudvig 2011). These seed mixes create communities that may be resilient

47   to changes in nutrient availability (Craven et al. 2016), competition from non-natives (Funk et al.

48   2008; Oakley & Knox 2013; Yurkonis et al. 2012; Norland et al. 2013), and climate change

49   (Isbell et al. 2015). Evolutionary theory emphasizes the important role both inter- and

50   intraspecific variation established within seed mixes may have to restoration success over time

51   (McKay et al. 2005). Greater biodiversity within restoration communities may increase total

52   plant productivity across time leading to increased stability in soil nutrient availability (Craven et

53   al. 2016), and resilience to extreme events (Isbell et al. 2015). In addition, intraspecific variation

54   is essential as this may provide the raw material that natural selection may act upon and is

55   needed to maintain species' evolutionary potential (Pizza et al. 2021; McKay et al. 2005).

56   Despite the importance of intraspecific diversity to restoration success, few studies have

57   quantified the role diversity within species has to restoration outcomes (Hamilton et al. 2020).

58   Consequently, to ensure that plant communities persist over time and in response to change,

59   there is a need to consider both the role of within and between species diversity to restoration.

60   Current strategies used to establish seed mixes often advocate a 'local is best' approach

61   (Broadhurst et al. 2008; McKay et al. 2005). This approach assumes that local seed sources will

62   have greatest fitness in local restoration environments relative to non-local sources (Kawecki &

63   Ebert 2004; Hoban et al. 2016). While there is evidence of local adaptation for many plant

64   species (Leimu et al. 2010; Hereford 2009), the degree or scale of adaptation is often unknown

65   (McKay et al. 2005). Furthermore, to conserve evolutionary potential requires genetic variation

66   (Kawecki & Ebert 2004). Genetic diversity is the raw material that selection acts upon and is

67   necessary for adaptation to changing environmental conditions. Genetic variation may be lost

68   through random fluctuations in population size via genetic drift, but maintained through gene

69   flow among populations (Reed & Frankham 2003). In addition, small, isolated plant populations

70   that exhibit reduced connectivity or gene flow may exhibit reduced genetic variation, but

71   increased genetic differentiation (Durka et al. 2017). If seeds are sourced locally for restoration

72   from small, isolated populations then individual seed sources may not have the requisite genetic

73   variation needed to adapt to change (Davis et al. 2005; Etterson & Shaw 2001). To ensure the

74   maintenance of evolutionary potential therefore may require seed sourcing strategies that

75   increase genetic diversity. Accounting for the role evolutionary forces play in the maintenance of

76   diversity will aid in establishing seed mixes that ultimately increase restoration success

77   (Bucharova et al. 2017; Hamilton et al. 2020).

78   To ensure preservation of evolutionary potential, variation within species is required

79   alongside the establishment of species rich seed mixes. The combination of intraspecific and

80   interspecific species diversity can influence community composition during establishment

81   (Larson et al. 2013). Diversity at these two scales can impact short-term response to the

82   environment and competition with local seed banks (Grman et al. 2013). During the first few

83   years following restoration it is expected that communities will be largely dominated by non-

84   seeded weedy species typically found within the soil seed bank (Bakker et al. 1996). For

85   example, when comparing an active prairie restoration to multiple remnant prairies, Martin et al.

86   (2005) observed more non-native species present within the restoration, with the overall

87   proportion of non-natives ranging from 236% to 413% higher in the restoration relative to

88   remnant sites. Thus, considering early establishment of seeded relative to non-seeded species

89   may be important to predicting longer-term plant community composition. Despite the potential

90   importance of early establishment to long-term restoration success, this phase is often

91   overlooked in favor of evaluating restorations after they have been established for several years.

92   Globally, native grasslands remain one of the most critically imperiled ecosystems

93   requiring active restoration (Hoekstra et al., 2005). These ecosystems provide essential services,

94   including maintenance of hydrological flow and retention (Seeling & DeKeyser 2006), carbon

95   sequestration (Euliss et al. 2006), nutrient cycling, and habitat for a diversity of species (Helzer

96   & Jelinski 1999; Skagen et al. 2008). Throughout the North American Great Plains, up to 87% of

97   historical grassland habitat has been lost primarily to agricultural conversion (Comer et al. 2018;

98   Hoekstra et al. 2005; Samson et al. 1999) leading to highly fragmented and isolated remnant

99   habitats. Where these grasslands remain, they are prone to invasion by non-native species and the

100   evolutionary consequences of isolation, which has lasting negative effects to diversity and

101   species richness (DiAllesandro et al. 2013; Haddad et al. 2015). Ensuring seed mixes restore

102   grassland populations so they have the capacity to adapt to change, resist invasion, and persist

103   over time is critical. However, the role of intraspecific diversity within seed mixes to restoration

104   success has yet to be empirically evaluated. Therefore, it is necessary to consider the impact of

105   both species and population diversity within seed mixes has to establishment of grassland

106   restorations.

107   We assessed plant community diversity following restoration using single- and multi-

108   source seed mixes to test the role within-species seed source diversity played in community

109   establishment. We used seed collected from five unique populations for each of 14 different

110   species as a proxy for creating genetic diversity within a seed mix. We expected that increasing

111   the number of unique seed sources per species used within a seed mix would lead to increased

112   emergence diversity following restoration relative to the use of a single seed source seed mix

113   (Bucharova et al. 2018). Overall, we predicted greater within-species diversity for seed mixes

114   would lead to increased species diversity in restored plant communities. This research

115   empirically evaluates the role of within species to between species diversity following

116   restoration. This study will provide a baseline understanding of the role of diversity across scales

117   to establishment during restoration.

118   METHODS

119   *Seed Collection*

120   In the summer of 2019, seed from 12 forb and two grass species were collected between

121   June and October from remnant native prairies within the Northern Great Plains of the United

122   States. A minimum of five unique populations per species each were collected from the Missouri

123   Coteau region of North and South Dakota and from the northwestern prairie region of Minnesota

124   (Table 1, Fig. 1). These 14 species were chosen because they are widely distributed throughout

125   the Northern Great Plains and are commonly used in regional restoration seed mixes (e.g., Smith

126   2010; Kurtz 2013). In addition, to control for potential dominance of warm-season grasses and to

127   increase establishment of sown forbs, species chosen were weighted toward forb species

128   (McCain et al. 2010; Norland et al. 2013; Dickson & Busby 2009). Populations were classified

129   as distinct if separated by at least one mile, however, were more commonly spaced further apart.

130   In northwestern MN, distances between seed source locations ranged from 3 km to 215 km

131   (Table S2), and pairwise distances between the restoration site and seed source ranged from 2 km

132   to 129 km (Table S3). Within the Missouri Coteau region, distances between seed source

133   locations ranged between 2 km and 312 km (Table S4), and pairwise distances between the

134   restoration site and seed source site ranged from 3.5 km to 214 km (Table S5).

135   Seed was hand-harvested as it ripened, with seed harvested multiple times at different

136   sites throughout the growing season following Bureau of Land Management seed harvesting

137   guidelines (BLM 2015). Within each population, individual maternal seed heads were sampled at

138   least three feet apart to reduce potential relatedness within populations. For species with multiple

139   seed heads, no more than 30% of available seed per maternal seed head was collected.

140   *Seed Mix Preparation*

141   Following harvest, seeds were dried at room temperature for a minimum of two weeks and

142   then transferred to 4°C storage for seven months to provide cold stratification and maintain

143   viability. Seeds were cleaned using several species-specific approaches. Large seeds were

144   stripped by hand, smaller seeds separated using sieves, *Hesperostipa comata* (Needle and thread

145   grass) seed awns were trimmed during the drying process to limit tangling, and *Solidago rigida*

146   (Stiff Goldenrod) and *Helianthus maximiliani* (Maximilian sunflower) and *H. pauciflorus* (Stiff

147   sunflower) seed were mechanically cleaned and separated using a Fractioning Aspirator Test

148   Model at the USDA Agricultural Research Center in Fargo, ND.

149   Seed was weighed for each species (Mettler Toledo, ML503T/00) from each population to

150   calculate population-specific numbers of seeds using a seeds per gram conversion (Table S1). To

151   maximize the seeds per species in the mix and ensure seed mix consistency across treatments and

152   replicates, the amount of seeds to include in the mix per species was calculated based on the

153   population with the lowest seed weight (g). In addition, for *Artemisia fringida* (Fringed

154   sagewort), *H. pauciflorus*, and *S. rigida,* the amount of seeds used in the seed mixes was reduced

155   by 0.9%, 3.5-7.0%, and 4.4-6.0% of the lowest seed weight respectively, to ensure these species

156   were not overrepresented in seed mixes as they can exhibit dominant characteristics (Table 1).

157   Across the two regions, seed mixes were established using the same species with the

158   exceptions of *A. fringida*, *Anemone cylindrica* (Tall timbleweed), and *Schizachyrium scoparium*

159   (Little bluestem), which were collected and planted exclusively in the northwestern MN region

160   and *Ratibida columnifera* (Prairie coneflower), *H. comata*, *Bouteloua gracilis* (Blue grama),

161   which were collected and planted exclusively in the Missouri Coteau region. Five different seed

162   mixes were established each using a single unique population per species for the seed mix within

163   each of the two restoration regions. For these single-source seed mixes, populations for the

164   different species were largely sourced from similar latitudes to minimize potential impacts

165   associated with latitudinal variation in phenology (Olsson & Ågren 2002; Dunnell & Travers

166   2011) (Fig. 1). In addition to five single source seed mixes, one multiple-source seed mix was

167   established for each region. The multi-source seed mix used proportionally the same amount of

168   seeds per species as the single-source mix, but each species' contribution was divided evenly

169   across five population sources. Thus, for both single and multi-source seed mixes the proportion

170   of seed used per species was the same. In this way, the ratio of species present within the single

171   source and multi-source was maintained across seed mixes for direct comparison. Vermiculite

172   (Vigoro) was added to final seed mixes in a 1:1 ratio as a common method to increase seed to

173   soil contact during planting and thus increase probability of emergence (Shaw et al. 2020).

174   *Seed Viability*

175   Unused seed from the restoration plots sampled from the northwestern MN region were sent

176   to South Dakota State University's Seed Testing Laboratory to assess seed viability. Unused seed

177   from the Missouri Coteau were not available for seed viability testing. These tests evaluated the

178   total viability of individual species when grown under ideal laboratory growth conditions to

179   induce germination. This test reported the percent of seed that germinated defined as the total

180   number of individuals emerged per seeds planted, percent of hard seed defined as seed that is

181   dormant due to a water impervious seedcoat, and dormant seed which is defined as seed that is

182   viable but does not germinate due to a physical or physiological condition (SDSU Seed Testing

183   Laboratory; https://www.sdstate.edu/sites/default/files/file-archive/2021-07/Seed-Testing-

184   Lab.pdf).

185   *Restoration Sites and Site Preparation*

186   During May and June of 2019, experimental restoration sites were identified and prepped

187   in both the northwestern MN and Missouri Coteau regions. The northwestern MN restoration site

188   was established at the Minnesota State University Moorhead Regional Science Center (RSC)

189   (46.872, -96.452) in Glyndon, MN. Portions of this site are abandoned agricultural brome fields

190   that are adjacent to remnant mesic prairie owned by Buffalo River State Park. Another portion of

191   this site was actively maintained as the Ponderosa golf course starting in 1962 and continued

192   operation after the transfer of ownership until May 2015, following which limited mowing

193   management has occurred. Due to site and space limitations, both areas of this site were used to

194   establish the experimental plots. The Missouri Coteau restoration site was established on the

195   Samuel H. Ordway Prairie Preserve (ORD) (45.704 -99.086), owned and managed by The

196   Nature Conservancy (TNC). Prior to TNC ownership in 1978, this site was used as a

197   brome/alfalfa production plot for cattle. Since TNC's ownership, this site has been maintained

198   for hay production every other year.

199   In 2019, the RSC site was prepared by placing landscape cloth over experimental

200   restoration plots to remove existing vegetation and limit potential establishment and competition

201   with the existing seedbed prior to applying the restoration treatment. In fall 2019, the ORD site

202   was treated with herbicide prior to application of restoration treatment (Roundup®, 3-4%

203   concentration) within each plot to reduce competition with existing weedy vegetation during

204   establishment. Additionally, all plots had a second Roundup treatment in early May, 2020 to

205   further reduce *Bromus inermis* (Smooth brome) encroachment.

206   At each site, twenty 3 x 3m experimental restoration plots were established. This included

207   establishment of five different single source seed treatment plots each replicated three times

208   (n=15) and one multi-seed source treatment replicated five times (n=5). For each individual

209   replicated plot within a seed treatment, a barrier of 3m was maintained and a minimum 100m

210   buffer maintained between each single- and multi-source seed treatment group to limit potential

211   gene flow between plots.

212   *Planting Experimental Restoration Treatments*

213   To establish the restoration treatments, tarps were removed from the plots at the RSC site,

214   and litter was raked and hand weeded in April 2020 at both sites to expose the seed bed.

215   Following this, each plot was broadcast seeded and then raked again to increase seed-soil

216   contact. For both sites, five times the total commonly recommended seeding rate of ~5kg (11

217   pounds) of seeds per acre were applied to increase probability of emergence success (Rowe

218   2010). Higher seeding rates were applied as these rates have previously been associated with

219   increased establishment and diversity following restoration (Sheley & Half 2006; Barr et al.

220   2017). An agri-fab push lawn roller was used to increase seed to soil contact and enhance the

221   probability of germination success. To limit potential carryover of seeds between seed treatments

222   the roller was rinsed and dried between each application. Finally, each plot received a one-time

223   watering treatment. Throughout the growing season, plot maintenance included weekly barrier

224   mowing around each plot. In July, mid-season mowing was performed at both sites to increase

225   light availability and reduce competition with non-seeded species (Maron & Jefferies 2001; Kaul

226   & Wilsey 2020). Plots were mowed at the maximum adjustable height setting (12.7cm) and all

227   trimmings were removed.

228   *Data Collection*

229   Each restoration plot was visited once per month at both sites between June and

230   September of 2020 to assess plant community composition. A 0.2m x 0.2m quadrat randomly

231   placed at each of the four cardinal corners and center of each replicated experimental plot was

232   used to estimate community composition of the broader restoration plot. To reduce the impact of

233   edge effects, quadrats were not placed directly at the edges of each plot. For all species present in

234   the quadrat, we counted the number of individuals present and estimated the percent cover per

235   species. Individuals that were unidentifiable in the field were marked with unique toothpicks and

236   photographed for later identification. There were two unknown species at the RSC site and three

237   at the ORD site that did not match planted species seedlings and were unable to be identified.

238   These species were uniquely labeled as unknowns and included in diversity calculations as

239   unique non-seeded species. Total percent cover of dead vegetation and percent bare soil cover

240   was also assessed visually within the quadrat. At the quadrat-level, total species coverage was

241   recorded as the total percent coverage of each species, litter coverage was the percent cover of

242   dead matter covering the ground, and soil coverage was the percent of visible bare ground. Each

243   coverage estimate was assessed with a modified Daubenmire cover-class system for grassland

244   vegetation (Table S6; Daubenmire 1959) and averaged across quadrats to obtain replicate-level

245   percent coverages for each seed mix treatment.

246   *Statistical Analysis*

247   To infer plant productivity and assess plant community composition following

248   restoration, species diversity metrics such as richness, evenness, abundance, and associated

249   diversity indices are often used and may be monitored over time (Martin et al. 2005; Polley et al.

250   2003). We tested for differences in community composition based on seed mix treatment at each

251   of our restoration sites using measures of species richness and diversity. Species richness was

252   defined as the total number of species present across all five quadrats sampled per replicate and

253   abundance as the total number of individuals present per species across quadrats. We also

254   analyzed the total number of unique species and the number of seeded species that established

255   within seed treatments for replicated plots. To evaluate our seed treatment communities

256   regardless of planted or non-seeded species status, we calculated Shannon's Diversity Index (H')

257   for each seed treatment and each replicate plot across time from June to September.

258
$$H' = \sum_{i=1}^{s} p_i \, ln p_i$$

259   Where *s* is the total number of species within the community (richness), p_i is the proportion of

260   each species (*i*) within the community relative to the total number of species multiplied by the

261   natural logarithm and summed across all species to get a value between 0-1. Values closer to 0

262   indicated lower diversity and values closer to 1 indicated higher diversity. We used Shannon's

263   Diversity for our data as it was the most appropriate given our data collection approach

264   (Magurran 2004). Diversity indices were calculated at the seed treatment level and for the

265   individual replicates within seed treatment to create distance matrices.

266   To compare plant community diversity within each restoration site for varying seed

267   treatments across time we used non-metric multidimensional scaling (NMDS) in three

268   dimensions with a Bray—Curtis dissimilarity distance matrix which was derived from the

269   Shannon's diversity indices for each seed treatment and each month of data collection. We used

270   NMDS because it uses an ordination approach where community data is summarized on two-

271   axes and communities that are more similar cluster together (Ruiz-Jaen & Aide 2005).

272   To evaluate differences between community compositions, we performed permutational

273   ANOVAs (PERMANOVAS) on the same Shannon's diversity values for seed treatment

274   communities across each month, using the adonis function in package "vegan" (Oksanen et al.

275   2020). We used a PERMANOVA approach to evaluate differences between individual seed

276   treatments and more broadly between single source and five-source community diversity. Seed

277   treatment, replicate, month, and the interaction of seed treatment and month were predictor

278   variables and the percent bare ground and thatch were included as random-effect variables within

279   our models. Post-hoc pairwise comparisons were performed to evaluate differences between seed

280   mix treatment per month for RSC communities and by month for ORD communities within the

281   pairwise.adonis function in package "pairwiseAdonis" (Martinez Arbizu 2019). All analyses

282   were conducted in R version 4.0.2 (R Core Team 2016).

283

284   RESULTS

285   *Seed Viability*

286   Six of the 14 species sent for testing had enough seed for an assessment of viability.

287   Variability in seed viability may impact how individual species may or may not establish within

288   the first year following restoration. Seeds from *Amorpha canescens* exhibited a viability score of

289   20% with 16% of seed reaching germination, 4% labeled as hard seed, and 0% dormant seed.

290   Seeds from *Anemone cylindrica* exhibited 82% viability with 75% of seed reaching germination,

291   0% hard seed and 7% assessed as dormant. Seeds from *Artemisia fringida* were 62% viable, with

292   25% of seed reaching germination, 0% labeled as hard seed, and 37% dormant seed. *Geum*

293   *triflorum* seed had a total viability of 47% with 47% of seed reaching germination, 0% labeled as

294   hard seed, and 0% dormant seed. *Potentilla arguta* seed exhibited 88% viability, with 66% of

295   seed reaching germination and 0% labeled as hard seed, 22% dormant seed. Finally, *Solidago*

296   *rigida* seeds had a viability score of 54% with 44% of seed reaching germination, 10% labeled as

297   hard seed, and 10% dormant seed.

298   *Plant Community Structure following Restoration*

299   Seed mix application at both the RSC and ORD sites resulted in a mixture of seeded and

300   non-seeded species emergence. At the RSC site, seeded species emerged from all plots excluding

301   seed treatment 'D' in the first growing season. Of seed mix treatment types, the multi-source

302   seed mix type 'ABCDE' had the greatest number of seeded species emerge, including *Echinacea*

303   *angustifolia*, *Helianthus maximilani*, and *Verbena hastata*. Across all seed treatments at the RSC

304   site, *Helianthus maximilani* exhibited the greatest rate of emergence, followed by *Liatris*

305   *punctata*. In the first year of observation, in total only five of the seeded species established at

306   the RSC site. At the ORD site, seeded species emerged within all plots in the first growing

307   season. Of seed mix treatment types, the multiple-source seed mix type 'ABCDE' and the single-

308   source seed treatment 'C' had the greatest number of seeded species emerge, including *H.*

309   *maximiliani*, *S.rigida* which were found within every seed treatment, followed by *Ratibida*

310   *columnifera*, and *Dalea purpurea*. In total only six unique seeded species established at the ORD

311   site.

312   At both restoration sites, seed treatment plots were largely dominated by non-seeded

313   species (Fig. 2.) At the RSC site the most common species within our experimental restoration

314   plots were *Ambrosia psilostachya* (Western Ragweed)*, Melilotus sp.* (Sweetclover sp.), *Panicum*

315   *capillare* (Witchgrass), *Poa pratensis* (Kentucky Bluegrass), *Oxalis stricta* (Yellow Wood

316   Sorrel), *Trifolium repens* (White Clover). At the ORD site the most common species within our

317   experimental restoration plots were *A. absinthium*, *Bromus inermis* (Smooth Brome), and *P.*

318   *pratensis*.

319   To evaluate plant community-level differences between seed mix treatment types and

320   across the growing season, we used a PERMANOVA based on Shannon's Diversity.

321   Additionally, to visualize any differences in these plant communities we used an NMDS with

322   Bray–Curtis dissimilarity. Within the RSC site, we found significant community-level

323   differences based on seed mix treatments (pseudo-F= 18.268, p = 0.001;), plot replicate (pseudo-

324   F =7.868, p = 0.001), month (pseudo-F= 2.677, p = 0.018), and the interaction of seed treatment

325   and month (*Pseudo-F*= 2.172, p = 0.008; Table 2). However, as very few seeded species

326   established across seed mix treatments, the differences observed appear to be largely driven by

327   site-level differences associated with spatial heterogeneity in the presence of non-seeded species

328   (Fig. 3B). To then evaluate which seed treatments, or location of seed treatments within the RSC

329   site were compositionally different, we then performed individual pairwise analyses. Pairwise

330   comparisons evaluating community compositions differences across seed mix treatments were

331   subset by month to account for the significant interaction of seed treatment and month found

332   within our PERMANOVA results. From these comparisons we found the five-source seed

333 treatment was significantly different from all single-source seed treatments across all months

334 with the sole exception of seed source 'E, which became more similar to the five-source

335 treatment over time (Table S7). This follows our expectation that the multiple-source treatment

336 would produce a more diverse community when compared to single-source treatments; however,

337 with the caveat that differences observed seem to be driven largely by the diversity of non-

338 seeded species present within individual plots.

339 Within the ORD experimental restoration site, we found no significant community-level

340 differences between seed mix treatments. However, within our PERMANOVA of community

341 composition based on Shannon's Diversity Index, we observed a significant difference among

342 our ORD communities based on month alone (pseudo-F= 0.385, p<0.001; Table 3). These results

343 indicate that any differences in community diversity was not due to seed mix treatments but were

344 primarily explained by the growing season (Fig. 3A). Pairwise comparisons found that plant

345 community composition in June was significantly different from the later seasonal communities

346 in August and September (Table S8). These results suggest that community diversity observed

347 across the restoration site was different in June than was observed later in the season.

348 DISCUSSION

349 Current local seed sourcing approaches during restoration may not adequately incorporate

350 within species genetic diversity needed to re-establish functional plant communities for

351 adaptation to changing environmental conditions. Thus, establishing diversity within and

352 between species for seed mixes will be critical to ensuring restoration success. Using seed source

353 as a proxy to indicate increased genetic variation, we have empirically evaluated how community

354 diversity establishes following the use of single and multiple- source seed mix treatments. There

355   was no major effect of seed mix treatment type on increasing community diversity within the

356   first year following restoration at two sites. However, community diversity across seed mix

357   treatment types at this early stage following restoration was strongly influenced by spatial

358   heterogeneity and by the growing season across the RSC restoration site, and strongly influenced

359   by time at the ORD site. Community diversity within both sites was largely dominated by non-

360   seeded species, with limited emergence of seeded species within the first year. These

361   observations are consistent with previous restoration studies, which observed that non-seeded

362   species may dominate restored environments during the first several years following restoration

363   (Kaul & Wilsey 2020). Although no differences were observed in community diversity between

364   our single and multiple-source seed mix treatments, our results suggest that first-year restoration

365   communities are influenced by heterogeneity in a restoration site and temporally by the growing

366   season. Thus, land-use history may be important in influencing plant establishment and

367   persistence over time, particularly in the first year following restoration.

368   *Seed Viability*

369   Although non-seeded species were expected in the first year, variation in seed viability

370   within our seed mixes (ranging from 20-88% for the RSC site) may have impacted first year

371   emergence. For seed viability testing, 7-37% of seeds were considered "dormant" and therefore

372   may have germinated within the first year, but could emerge in subsequent years provided that

373   environmental conditions in the future are favorable for germination. In addition, seed predation

374   and seedling herbivory may have reduced establishment success during the first year. Herbivore

375   disturbance can mediate non-seeded species dispersal through selective seed herbivory on native

376   plant species (Howe & Brown 2000) which may affect overall species diversity. At the

377   northwestern MN site, the thirteen lined-ground squirrel (*Ictidomys tridecemlineatus*) was

378   observed, alongside nearby and within-plot gopher mounds. As our study design was aimed to

379   mimic natural restoration practices, we did not take measures to actively exclude mammals from

380   the restoration sites, but instead used approximately five times the standard seeding rate for each

381   seed mix treatment type. High seeding rates are often used to mitigate potential effects of seed

382   viability and herbivory on seedling establishment, and increase overall plant densities

383   (Applestein et al. 2018).

384   *Plant Community Structure following Restoration*

385   We compared species richness following restoration with seed mixes containing a single

386   source per species or multiple sources per species across two restoration sites. Multi-source seed

387   mixes were associated with greater seeded species richness at the RSC site, but not the ORD

388   restoration site. In the first growing season following the restoration four times the number of

389   non-seeded species were observed compared to seeded species at the ORD site, and seven times

390   at the RSC site, respectively (Fig. 2). This is consistent with rates observed previously in

391   grassland restoration experiments (Martin et al. 2005). Seeded species that emerged were those

392   have evolved traits that provide competitive advantages in grassland ecosystems, such as

393   rhizomatous root systems (Mangan et al. 2011; Dickson & Busby 2009) or mutualist fungal

394   relationships which can promote and facilitate establishment (Busby et al. 2011). For example,

395   *H. maximiliani* is a widespread perennial forb native to prairies in the United States and Canada

396   (USDA). *H. maximiliani* readily established at both sites across seed treatments and is often

397   found in remnant and restored prairies as a sub-dominant or dominant species (Dickson & Busby

398   2009). Previous studies have found that *H. maximiliani* is often one of the most productive forb

399   species within plant communities as it may outcompete other species due to its rhizomatous root

400   system that creates a spreading pattern for nutrient uptake, and thick sprouting stem that leads to

401   increased biomass production and vegetative coverage (McKenna et al. 2019; Mangan et al.

402   2011; Dickson & Busby 2009). *Ratibida columnifera* was another common perennial species to

403   establish at the ORD site and across various seed treatments. This species occurs widely

404   throughout southern Canada, across the US Great Plains, and into Northern Mexico (USDA). In

405   previous experiments, *R. columnifera* has been observed to have high first year survival and a

406   life span around three years and may negatively impact the abundance of other forbs (Lauenroth

407   & Adler 2008; Dickson & Busby 2009). The competitive advantage expressed by *R. columnifera*

408   may be due to its establishment through a prominent taproot and strong positive relationship with

409   arbuscular mycorrhizal fungi which aids nutrient uptake and growth (Busby et al. 2011). In

410   addition, both species are native to our study regions, thus may exist within the seed bank

411   currently. However, during field site visits we did not observe *H. maximiliani* at either site

412   outside of the experimental plots. *Ratibida columnifera* was present within the RSC site but was

413   not included in the experimental seed mixes and was not present within the plots. Evaluating

414   what species readily establish during the early stage of a restoration may aid in future seed mix

415   design choices to combat non-native species establishment, and to ensure early restoration

416   success.

417   Both the PERMANOVA and NMDS analyses assessed plant community structure using

418   measures of diversity from seeded and non-seeded species quantified across seed mix treatments

419   for each site. For RSC, the seed treatment with the most diverse community established

420   throughout the season was our multiple-source mix (ABCDE). The multi-source seed treatments

421   were planted on the portion of the site that was once a golf course, near a remnant mesic area

422   with surrounding woody vegetation. Several species that established solely within this treatment

423   were persistent within the woody vegetation nearby, including *Achillea millefolium*, *Plantago*

424   *major, P. annua,* and *Salix interior*. The presence of these species only within our multiple-

425   source treatment plots is therefore likely influenced by the neighboring community, although as

426   predicted this treatment had the most seeded species establish. This treatment was significantly

427   different from all other seed mix treatments, except 'E' which was compositionally more similar

428   during later seasonal months (Table S7). Given the spatial proximity of the 'ABCDE' and 'E'

429   treatments, similar communities likely arose due to local site conditions, including below ground

430   nutrient resources and varying seed banks across the site. Community composition at RSC also

431   varied over time in response to seed mix treatments (Table 2). However, the spatial differences

432   observed in community composition were maintained throughout the growing season.

433   Although multi-source seed mixes were associated with greater sown species richness

434   within the RSC plots, total sown species richness was greater across all ORD plots, but not

435   different across seed treatments (Fig. 2). The increase in total seeded species richness could

436   indicate there was less competition from non-seeded species which may allow for increased

437   establishment, or seeded species already existed within the soil seed banks. Although seed

438   treatment did not appear to influence sown species establishment within ORD plots, growing

439   season influenced communities with similar community diversity establishing throughout the

440   growing season (Fig. 3b). Pairwise comparisons of community diversity across time indicated

441   that June was the only month that was significantly different from the community present in later

442   months. This may indicate that early season emergence drives the formation of community

443   structure across time. These data provide a baseline understanding of site-specific community

444   diversity to monitor composition change over time and across seed treatments.

445   Comparison across sites suggests the different patterns of diversity and those factors that

446   structure diversity across sites are likely associated with different land-use histories. The

447   experimental seed mix treatments at the ORD restoration site were established on an old

448   agricultural field with active management for hay production. The site has experienced similar

449   land-use history, which has likely largely homogenized the above and belowground plant

450   community, currently dominated by smooth brome (*B. inermis*) and alfalfa (*Medicago sativa*).

451   The influence of agricultural activity and dominance of smooth brome and alfafa has also likely

452   contributed to further homogenization of the associated seed bank, reducing richness and

453   diversity of the non-seeded species community (Bekker et al. 1997). In contrast to the

454   homogeneity observed at the ORD site, the land-use history at RSC was more heterogeneous,

455   which may have contributed to spatial variation in plant community establishment across the site.

456   Interestingly, while the ORD community structure did not exhibit differences associated with

457   seed mix treatment, the RSC site did exhibit significant differences across seed mix treatments.

458   Single-source seed treatments A, B, and C were established on a portion of the site that was once

459   planted with brome and alfalfa for haying purposes. In contrast, seed treatments D, E, and the

460   multiple-source mix ABCDE were established on a portion of the site that was a golf course up

461   until 2015. Combined, land use history and varying impacts of the seed bank and nutrient profile

462   across the site suggests there is substantial heterogeneity across the site that may have influenced

463   emergence following application of seed treatments. Despite site preparation methods used to

464   prevent non-seeded species establishing within plots these differences may be reflected in the

465   site-level differences as opposed to seed mix application. Thus site-level differences are due to

466   spatial heterogeneity within the soil seed bank and nutrient availability associated with land-use

467   history impacting community establishment regardless of seed mix treatment.

468   Land use history can play an important role influencing how restoration communities

469   establish over time (Cousins et al. 2009; Grman et al. 2013). Spatial heterogeneity across a

470   restoration site could influence soil nutrient resources across the site and the associated species

471   that may persist within the seed bank (Ricklefs 1977; Bakker et al. 2003). Where greater nutrient

472   loading is observed, increased competition and exclusion between seeded and non-seeded

473   species for resources could be observed (Eskelinen et al. 2021; Stotz et al. 2019). Aggressive

474   non-seeded species often outcompete natives along nutrient load gradients leading to a

475   subsequent loss of available soil nutrient resources. This can have substantial impacts to native

476   plant diversity both above and belowground (Stevens & Carson 2002; Wilson & Tilman 1993;

477   Eskelinen et al. 2021). Thus, heterogeneity in the soil nutrients or lack thereof likely impacted

478   how communities established at both sites, but data on emergence provide a baseline to monitor

479   how patterns in community composition may change over time.

480   Although we were interested in which seeded species established within our seed mix

481   treatments, non-seeded species may also be important components to consider when evaluating

482   these experimental communities over time. In a previous study Kaul & Wilsey (2020) noted that

483   non-seeded weedy species abundance was the strongest predictor of species richness and

484   diversity in grassland restorations, regardless of the age of the restoration. The most common

485   non-seeded species to establish within our communities were introduced species, including cool-

486   season grasses *B. inermis* and *Poa pratensis*. These species typically outcompete natives for

487   resources, including both nutrient and light availability (reviewed in D'Antonio & Meyerson

488   2002). *Poa pratensis* establishes early in the spring before many native forbs, thus early

489   establishment and the consequent increased growing season may provide a competitive

490   advantage over native species (DeKeyser et al. 2015). *Bromus inermis* also establishes readily in

491   the spring and is a commonly planted pasture grass that readily forms a quickly establishing

492   monoculture through a rhizomatous root system (Stotz et al. 2019). The aggressive establishment

493   of *B. inermis* often leads to outcompeting and displacing native species which may lead to

494   decreased plant diversity and community homogenization of a site when it becomes an

495   established invader (Stotz et al. 2019; DiAllesandro et al. 2013). The prevalence of these well-

496   known invasive species within our treatments, despite our pre-seeding site prep to limit non-

497   seeded species establishment may indicate that more work is needed to successfully limit and

498   manage their establishment during restoration. Considering how these species establish may be

499   critical to restoration success as it may require more effort to shift these communities back to

500   native species (Martin & Wilsey 2014). Additionally, genetic variation within seeded species

501   used within seed mixes may mitigate some of the negative impacts of invasives. Genetic

502   variation may increase the diversity of genotypes that establish increasing the probabilities of

503   producing a self-sustaining, persistent population that can evolve over generations. Evaluating

504   which non-seeded species establish and tracking their abundance in the early stages of a

505   restoration will help guide restoration expectations and community management practices over

506   time.

507   Single versus multiple source seed mix treatments did not have an impact on community

508   composition diversity in the first year of restoration establishment. Our results suggest that early

509   emergence and diversity within a plant community following restoration is largely influenced by

510   land-use history. In addition, first-year emergence following restoration may be largely

511   insensitive to seed mix type if non-seeded species in the seedbank are able to outcompete seeded

512   species during establishment. Previous studies have shown that first year emergence positively

513   influences seeded species abundance and richness several years following restoration (Applestein

514   et al. 2018; Geaumont et al. 2019) Thus, while there is some evidence to suggest seed mix type

515   may impact the diversity of established species, long-term assessments over multiple years will

516   be necessary to quantify the full impact of seed mix type has to community diversity and

517   restoration success over time. Evaluating what seeded and non-seeded species establish in the

518   first year of a restoration will help inform future restoration plans for long-term restoration

519   success. Indeed, identifying those seeded species that may have the competitive ability to readily

520   establish may be needed during the design of seed mixes, both identifying those species that

521   should be included and the proportion of seed that may be necessary to maintain those species

522   over time.

523   Understanding the role within and among population genetic variation has on native

524   grassland restorations may have substantial implications to seed mix design recommendations.

525   We assumed here that a multi-population seed mix reflects increased genetic variation, however,

526   the degree to which population sources impact standing genetic variation within seed mixes

527   remains to be tested. Future work should include a genetic analysis of populations in single and

528   multi-source seed mixtures. Finally, although initial establishment results may be important to

529   early restoration success, longer-term monitoring will be necessary to evaluate the impact seed

530   mix treatment may have to community structure over time. Combined, genetic analysis and

531   longer-term monitoring of seed mix treatments will provide information needed for land

532   managers to establish seed sourcing guidelines critical to restoration in a changing environment.

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542   Fund, and The Nature Conservancy. The authors declare no conflict of interest. Data

543   accessibility will be provided.

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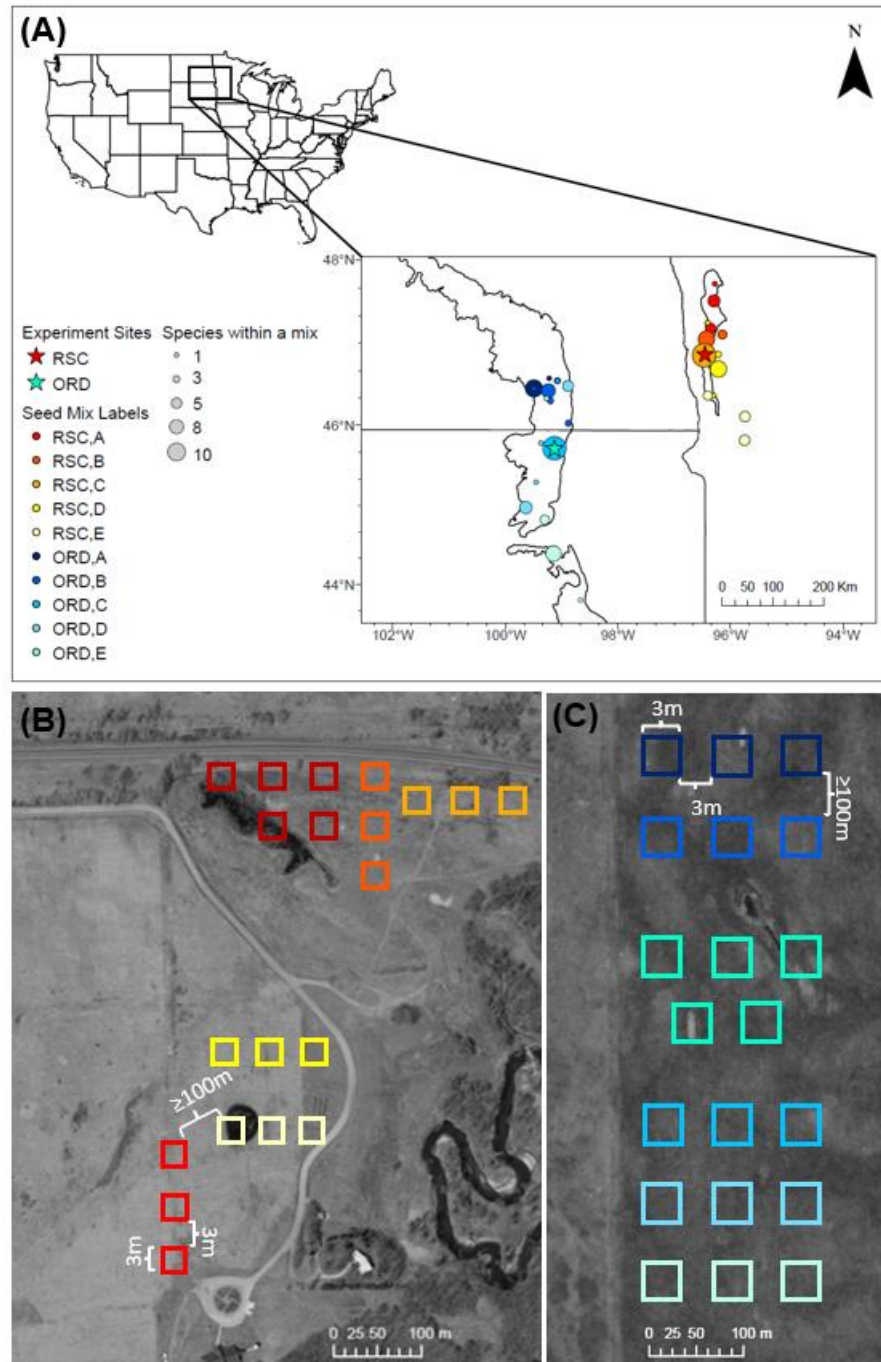
Fig. 1. A) Seed collection sites for seed mix treatments for Missouri Coteau (blues) and northwestern MN (reds) regions respectively. Colors represent individual seed mixes, and proportional symbols indicate the number of species sourced from a single site used within a seed mix. Stars indicate experimental site locations. B) Seed mix treatment layout at the RSC restoration site in Glyndon, MN. C) Seed mix treatment layout at ORD restoration site in Leola, SD. Colors correspond to seed treatment, single-source treatments include three replicate plots and the multiple-source treatment includes five replicate plots.

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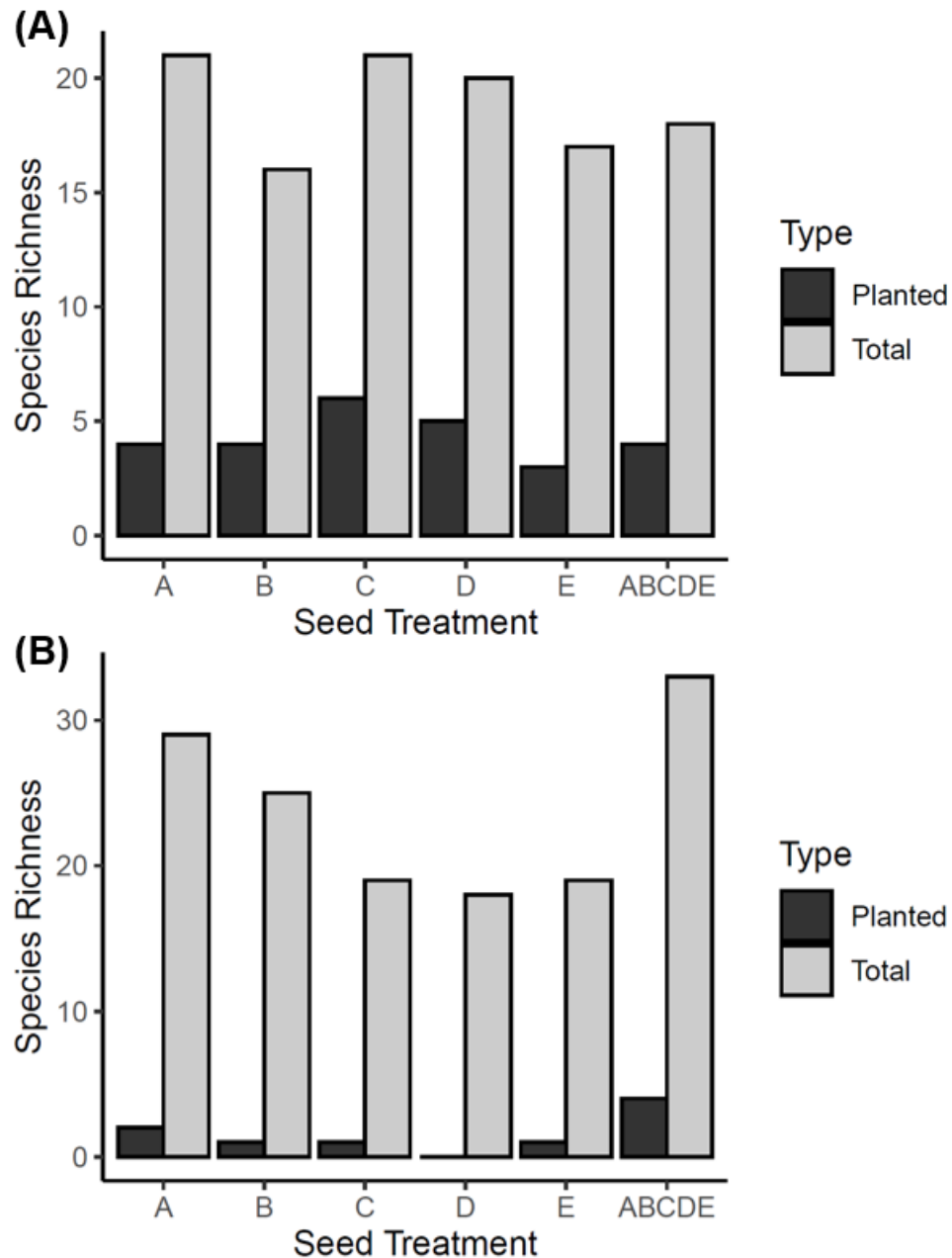
Fig. 2. Comparison of planted and total species richness within each seed mix treatment throughout June-September 2020 within the ORD experimental site (A) and RSC experimental site (B). Overall planted richness was greater in within all ORD seed treatments compared to RSC. Total species richness was higher in RSC than in ORD, and the multiple-source seed treatment, labeled ABCDE, had greatest planted species richness.
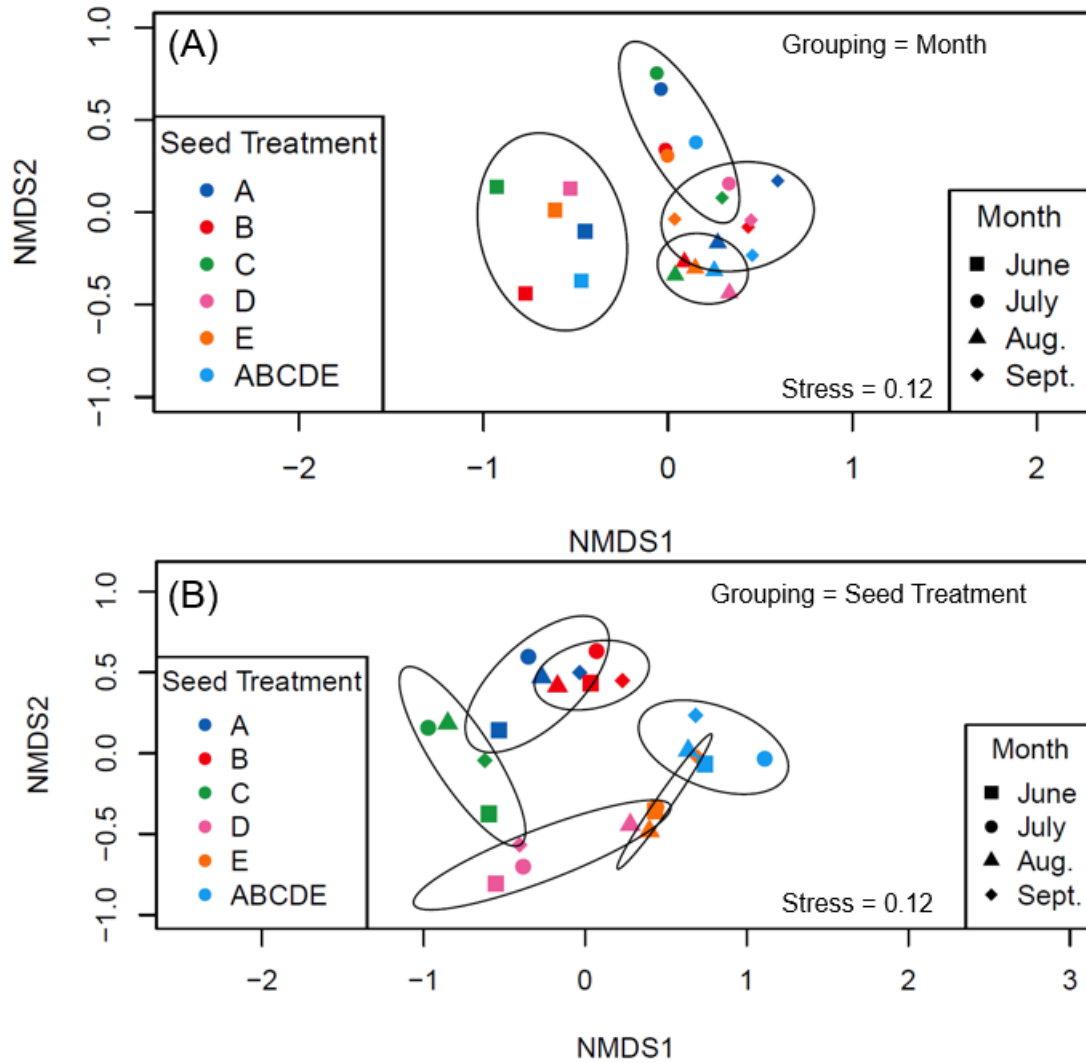
Fig. 3. Nonmetric Multidimensional Scaling with Bray – Curtis dissimilarity graphs of the first year established communities within (A) RSC communities grouped by seed treatment and (B) ORD communities grouped by month. Seed treatment indicated by color and shapes indicate month of data collection. Ellipses are 95% confidence intervals.

686 Table 1. Species used experimental restoration plots for RSC and ORD sites, weighed amounts used in individual seed mix treatments,
687 individual species composition within seed mixes, approximate seeds/m^2, and seed viability included where applicable.

| | RSC | | | | | ORD | | | |
Species Scientific Name	Single-population Seed Mix (g)	Five-population Seed Mix (g)	Species composition in mix (%)	Seeds/m^2	Viable Seed (%)	Single-population Seed Mix (g)	Five-population Seed Mix (g)	Species composition in mix (%)	Seeds/m^2
Amorpha canescens	12.5	2.5	21.8	784	20	12.5	2.5	23.2	784
Anemone cylindrica	7.5	1.5	13.1	764	82	-	-	-	-
Artemisia frigida	0.5	0.1	0.9	556	62	-	-	-	-
Bouteloua curtipendula	5.0	1.0	8.7	233	-	1.5	0.3	2.8	70
Bouteloua gracilis	-	-	-	-	-	0.5	0.1	0.9	78
Dalea purpurea	5.0	1.0	8.7	353	-	5.0	1.0	9.3	353
Echinacea angustifolia	8.0	1.6	13.9	219	-	8.0	1.6	14.8	219
Geum triflorum	1.3	0.3	2.2	132	47	8.5	1.7	15.8	899
Helianthus maximiliani	1.3	0.3	2.2	64	-	1.0	0.2	1.9	51
Helianthus pauciflorus	2.0	0.4	3.5	31	-	2.0	0.4	3.7	31
Hesperostipa comata	-	-	1.5	-	-	0.6	0.1	1.2	18
Liatris punctata	4.3	0.9	7.4	117	-	1.0	0.2	6.5	274
Pediomelum argophyllum	2.5	0.5	4.4	88	-	3.5	0.7	1.9	27
Potentilla arguta	1.5	0.3	-	1352	88	1.3	0.3	6.5	123
Ratibida columnifera	-	-	2.6	-	-	2.5	0.5	2.3	1127
Schizachyrium scoparium	2.8	0.6	4.8	162	-	-	-	4.6	412
Solidago rigida	2.5	0.5	4.4	402	54	2.5	0.5	4.6	402

688

689   Table 2. PERMANOVA results for community composition differences within RSC
690   experimental seed mix treatments, using Seed Treatment, Plot Replicate, Month, and the
691   interaction between seed treatment and month as main explanatory variables.

692

	Df	SS	MS	Pseudo F	R2	*P*
Seed Treatment	5	1.475	0.295	18.268	0.323	*0.001*
Plot Replicate	14	1.778	0.127	7.868	0.390	*0.001*
Month	3	0.130	0.043	2.677	0.028	*0.018*
Bare Ground	1	0.002	0.002	0.135	0.000	0.897
Thatch	1	0.007	0.007	0.408	0.001	0.640
Treatment:Month	15	0.526	0.035	2.172	0.115	*0.008*
Residuals	40	0.646	0.016		0.142	
Total	79	4.5627			1	

693
694
695   Table 3. PERMANOVA results for community composition differences within ORD
696   experimental seed mix treatments, using Seed Treatment, Plot Replicate, Month, and the
697   interaction between seed treatment and month as main explanatory variables.

	Df	SS	MS	Pseudo F	R2	*P*
Seed Treatment	5	0.181	0.036	2.165	0.077	0.068
PlotRep	14	0.228	0.016	0.975	0.097	0.509
Month	3	0.908	0.303	18.095	0.385	*0.001*
Bare Ground	1	0.018	0.018	1.079	0.008	0.314
Thatch	1	0.001	0.001	0.061	0.000	0.928
Treatment:Month	15	0.352	0.023	1.401	0.149	0.157
Residuals	40	0.669	0.017	0.284		
Total	79	2.358	1.000			

698