Short Title: Inundation suppresses fungi in *Typha.*

Title: Suppression of root-endogenous fungi in persistently inundated *Typha* roots.

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ABSTRACT

Wetland soils are defined by anoxic and reducing conditions that impose biogeochemically hostile conditions on plant roots and their endogenous fungal communities. The cosmopolitan wetland plant *Typha* L. mitigates root-zone anoxia efficiently, such that roots of these plants may constitute fungal habitats similar to roots in subaerially-exposed soils. Alternatively, fungi may compete with plant cells for limited oxygen in inundated roots. We hypothesized that extrinsic environmental factors may reduce fungal incidence and affect fungal community structure within inundated roots as compared to those in subaerially-exposed soils. We sampled roots of *Typha* spp. plants across inundation gradients in constructed reservoirs; root subsamples were microscopically examined for fungal structures, and morphologically-distinct fungal endophytes were cultured and isolated from surface sterilized subsamples. We found that the incidence of fungal hyphae was suppressed for all types of vegetative mycelia when roots were inundated, regardless of depth, but that there were no obvious differences in community composition of fungi cultured from roots growing in inundated vs subaerially-exposed soils. This suggests that the suppression of hyphae we observed in root samples did not result from changes in community composition. Instead, low hyphal incidence in inundated *Typha* roots may reflect germinal inhibition or unsuccessful initial colonization, possibly owing to plant-mediated redox dynamism in the surrounding soil. No variation was seen in the incidence of asexual spores, or chytridiomycetes, nor were there significant differences between geographically disparate sampling sites. Communities of root-endogenous fungi may therefore be influenced more strongly by external environmental factors, than by the environments that plant roots comprise.

KEY WORDS

aquatic macrophytes, dark septate fungi, endophytes, hydric soil, root-associated fungi, wetlands

INTRODUCTION

Root-endogenous fungi are complex communities of mutualists, commensals, parasites, and pathogens that are inhabit living vascular plant roots. Their incidence and diversity is a function of complex interactions between biotic and abiotic factors, including host taxonomy (Stevens et al. 2011), soluble carbon availability (Jones et al. 2009), soil nutrient availability (Johnson 1993), geography (Higgins et al. 2007), and climate (Newsham et al. 2008). In some instances, plants living under extreme levels of abiotic stress host root-endogenous fungi that may have beneficial or protective effects upon their hosts; in other cases some root-endogenous fungi may become deleterious (Mandyam and Jumpponen 2005, Rodriguez et al. 2009). Environments inside stressed plant roots can limit fungal growth, or impact which species of fungi can persist in plants growing in saline (Carvalho et al. 2003) or highly acidic soils (An et al. 2008), or in the presence of phytotoxic metals (Entry et al. 2002). By understanding the incidence and diversity of root-endogenous fungi in extreme environments, and how these fungi vary in response to abiotic stressors, we stand to gain considerable insight into their physiological traits.

Wetland soils are among the most biogeochemically challenging environments that vascular plants and root-endogenous fungi inhabit (Pezeshki and Delaune 2012). Prolonged inundation imposes a suite of interlinked abiotic stressors: inundated soils are anoxic and therefore reducing chemical environments, which influences availability of limiting nutrients and causes phytotoxic metals and organic acids to accumulate in the rhizosphere (Ponnamperuma 1984, Weis and Weis 2004, Reddy and Delaune 2008). Within the root environment, aerobically-respiring fungi compete with host cells for diminishing oxygen. Declining oxygen availability causes plant cells to switch from oxidative respiration to fermentation pathways (Vartapetian and

Jackson 1997, Gibbs and Greenway 2003, Greenway and Gibbs 2003), thereby also diminishing soluble carbon that might otherwise be available to root-endogenous fungi. Owing to the anoxic and reducing conditions that predominate in most wetland soils (Reddy and Delaune 2008), root-endogenous fungi historically have been considered minor contributors to wetland soil ecosystems (Khan and Belik 1995).

Most early work on root-endogenous fungi in both terrestrial and aquatic systems focused on arbuscular mycorrhizal fungi (AMF), but in recent decades it has become obvious that parasites, pathogens, and asymptomatic endophytic fungi also occur regularly in plant roots. Arbuscular mycorrhizal fungi comprise a mucoromycotan subphylum (Spatafora et al. 2016), and engage in obligate mutualisms with members of every vascular plant lineage that inhabits subaerially-exposed soils (Feijen et al. 2017). Although AMF have been reported as rare or absent in wetlands (e.g., Khan and Belik, 1995), but it has become apparent that AMF are common, occurring in roots of wetland plants that range from fully submerged to emergent (e.g., Søndergaard and Laegaard, 1997, Turner et al. 2000, Beck-Nielsen and Madsen 2001, Cornwell et al. 2001, Šraj-Kržič et al. 2006, Sudová et al. 2011, Wang et al. 2011, Zhang et al. 2014). Similarly, dark septate endophytes (DSE), a phylogenetically heterogenous guild of potentially mutualistic or weakly parasitic fungi that inhabit plant tissues without obvious host response (Mandyam and Jumpponen 2005, Mandyam et al. 2013), are observed frequently in roots of wetland plants (Cooke and Lefor 1998, Weishampel and Bedford 2006, Kai and Zhiwei 2006, de Marins et al. 2009, Sudová et al. 2011, Kohout et al. 2012). So too are true pathogens (Evans and Reeder 2000). Genetic assays corroborate our contemporary understanding of wetland plant roots as environments with highly diverse fungal communities (Kohout et al. 2012, Sandberg et al. 2014, You et al. 2015).

The ubiquity of fungi in roots of wetland plants may reflect the ability of these plants to mitigate anoxia within their roots. Plants that thrive in wetlands employ a variety of physiological and anatomical adaptations to mitigate anoxia (Strand 2002, Gibbs and Greenway 2003, Greenway and Gibbs 2003, Evans 2004, Colmer and Voesneck 2009). Many of these strategies involve active or passive aeration of roots (Strand 2002, Evans 2004). Some wetland plants are so effective at oxygenating submersed roots that there is abundant extra-radicle leakage of oxygen into surrounding sediments (Aldridge and Ganf 2003, Pezeshki and Delaune 2012). Well-aerated roots of wetland plants may thus resemble roots growing in subaerially-exposed soils, in terms of constituting suitable habitat for root-endogenous fungi. Conversely, the effectiveness of plants' mitigation strategies declines with depth, as reflected by differences in plant communities along depth gradients in wetlands (Spence 1982, Brix et al. 1992, Lemoine et al. 2012). Thus root-endogenous fungal communities may experience seasonal or persistently hypoxic to anoxic conditions in many cases. ts, Few studies have assessed the extent to which deeply inundated wetland fungal communities resemble those in subaerially-exposed soils (Stevens et al. 2010, Sandberg et al. 2014, Xu et al. 2016), even though such abiotic conditions are not paralleled in most terrestrial root environments

Here, we explored the incidence of root-endogenous fungi and their community structure in the cosmopolitan wetland plant *Typha* L., and tested the extent to which fungi inhabiting roots of *Typha* spp. vary across inundation gradients. *Typha*, colloquially known as cattails or reedmace, are cosmopolitan emergent macrophytes that span the entirety of inundation gradients. *Typha* spp. have been used as model plants in inundation studies (Ray and Inoue 2006, Inoue and Tsuchiya 2009). They employ pressurized convective ventilation to mitigate root hypoxia (Brix et al. 1992, Bendix et al. 1994, Tornberg et al. 1994), which is one of the most efficient aeration

strategies (Sorrell and Hawes 2009). Nevertheless, oxygen diffuses readily out of roots in reducing conditions (Kludze and Delaune 1996), and most oxygen within roots is consumed by plant respiration (Bedford et al. 1991, Chabbi et al. 2000). Some *Typha* species even exhibit increased metabolic oxygen demand when inundated (Matsui and Tsuchiya 2006). Actively growing fungi are thus in direct competition with host plant cells for diminishing oxygen. As such, we broadly hypothesized that *Typha* roots in subaerially-exposed soils would be more hospitable to aerobically respiring fungal endophytes than those at depth, which may experience more frequent or longer periods of hypoxia. To determine the effect of inundation (and declining oxygen availability by proxy), we assessed the incidence of fungal structures within roots to determine if their abundance differed across inundation gradients. We also assessed whether the community structure of culturable root-endogenous fungi differed across the gradients. Our study was replicated at three geographically disparate locations to account for potential geographic differences in fungal diversity and basin hydrology.

Our specific hypotheses and associated predictions are: (i) Under increasing inundation, plant roots will become increasingly inhospitable to aerobically respiring fungal endophytes. We predicted that the incidence of mycelial structures attributable to aerobically-respiring fungi would diminish commensurate with depth; structures associated with stress response, like sclerotia or conidia (asexual spores), would increase. Morphological structures attributable to facultative aerobes or anaerobes will not differ. (ii) Inundation will influence the composition of culturable fungal endophyte communities. We predicted that different morphotaxa will be cultured from roots grown in subaerially exposed soils versus those at depth. (iii) Some variation in incidence of structures or composition will be explained by geographic proximity alone (i.e. spatial autocorrelation). We predicted that fungal incidence and community composition of

samples within a reservoir would more closely resemble each other than those from other reservoirs.

MATERIALS AND METHODS

Organism, site selection and transect design. — Three species of *Typha* (*T. angustifolia*, *T. glauca* [*T. angustifolia* x *latifolia*], and *T. latifolia*) are present in the state of Kansas, and are known to readily hybridize (Kirk et al. 2011). We inventoried root-endogenous fungi in *Typha* spp. at the peak of flowering (early June, 2014) within three Kansas catchment basins: University of Kansas West Campus (38°56'58"N, 95°15'48.86"W), Cross Reservoir (39° 3'8"N, 95°11'2"W) and Melvern Lake Outlet (38°30'40"N, 95°41'59"W). All three reservoirs are drainage catchment basins constructed in limestone parent rock. They are dammed along their south aspect, and grassy vegetation atop the dam is mown throughout the growing season. Three transects were established along the dam in each reservoir, and extended from the deepest to highest incidence of *Typha* plants.

Sample collection and processing. — We excavated three plants along each transect: the most deeply inundated, the most subaerially-exposed, and at the midpoint of each transect (Fig. 1). We also compared fungi in *Typha* roots fungi with fungi in grasses growing in immediate proximity at the upper terminus of each transect (Fig. 1). Above-ground growth was removed in the field, and rhizomes with attached roots were sealed in individual sterile bags and transported to the laboratory on ice, where they were washed of sediment and then surface-sterilized (after Arnold et al. 2007) by sequential immersion in 95% EtOH (10 seconds), 10% sodium hypochlorite (2 min), and 70% EtOH (2 min). Root clippings were taken from each plant 5 cm below the divergence of stem from rhizome.

Incidence of fungal structures and culturable fungi in roots. — For each of three plants taken at every transect point (n = 108), we assessed the incidence of morphological structures by microscopic examination of *Typha* roots and by culturing endogenous fungi. Surface-sterilized root clippings for microscopic examination were stored in 95% EtOH at -20 °C, then cleared with 10% potassium hydroxide (KOH), fuchsin stained, and permanently mounted to glass slides using Eukitt mounting medium (O. Kindler GmbH). Following the root intersection method (McGonigle et al. 1990), roots of each plant were examined across 200 intersections for the presence of vegetative mycelia (comprising coenocytic/aseptate hyphae, hyaline septate hyphae, and dematiaceous hyphae) and other fungal structures (vesicles, conidiospores, or sporangia attributable to epi- and endobiontic chytrids). To assess community composition of culturable endogenous fungi, we aseptically plated surface-sterilized root clippings from each plant on potato dextrose agar (PDA) and V8 agar supplemented with ampicillin. Resultant fungi were isolated sequentially on PDA until pure cultures were obtained. Pure cultures were photographed, morphotyped (n = 83 different morphotypes), and archived in ultrapure (PCR-grade) H_2O. Cultures were deemed the same morphotype if colony morphology, growth, and colour were consistent on aerial and submersed (bottom) surfaces; singletons were removed from statistical analyses.

Statistical analyses. — Differences in the incidence of fungal structures were assessed via generalized linear mixed effects models (GLMMs) and generalized linear models (GLM) with negative binomial distributions. GLMs were marginally favoured under AIC, BIC, and Vuong's z-stat (Vuong 1989) in goodness of fit tests (Supplementary Data), however both the experimental design and null hypothesis expectations of spatial autocorrelation are better reflected using a random effect term that incorporates the transect point nested by reservoir.

Mixed effect multinomial logistic regressions for each type of fungal structure were implemented with the GLMER.NB function, which builds on GLMER, a component of the LME 4 1.1-18-1 package (Bates et al. 2015). We performed post-hoc general linear hypothesis testing of fitted models using multiple pairwise (Tukey) comparisons implemented with the GLHT function of the MULTCOMP package (Hothorn et al. 2008) in *R*. Explanatory significance of fixed and random effects was assessed with likelihood ratio tests.

To assess the community structure of 83 culturable root-endogenous fungal morphotypes, we performed nonmetric multidimensional scaling (NMS) ordinations in PC-ORD 6.08 (McCune and Mefford 2011). Ordination parameters tested 6 axes, and the dataset was sampled in 250 runs. A random number specified starting coordinates, and the stability criterion was set as 0.000001; stability was evaluated with 10 iterations, to a maximum of 500 iterations, stepping down in dimensionality with an initial step length of 0.20. Tie-breaking penalized unequal ordination distances (Kruskal's secondary approach). PERMANOVA tests of variance were conducted in PC-ORD, comparing experimental data against 10 000 random permutations.

RESULTS

Incidence of microscopic fungal structures was not linearly correlated with inundation. — All three types of vegetative mycelia (aseptate, hyaline, and dematiaceous hyphae) were most prolific in roots taken from subaerially-exposed soils at the highest transect points (Figs. 2A–C). Rather than diminishing with increasing depth as we predicted, the incidence of all hyphae instead only differed significantly between plants that were subaerially exposed and those from inundated soils, regardless of inundation depth (P = <0.05 for all Tukey pairwise comparisons, Figs. 2A–C). Non-hyphal fungal structures showed no significant differences between roots taken from inundated versus subaerially exposed soils. Vesicles were rare to absent in all *Typha*

roots (Fig. 2D), and neither the incidence of conidiospores (Fig. 2E) nor chytrids (Fig. 2F) was significantly different between samples (P = >0.05 for all Tukey pairwise comparisons, Figs. 2D–F). We tested inundation depth as a predictor of sample variance, and found it was not predictive of the incidence of chytrids ($\chi^2(3) = 2.62$, $P = 0.4549$), spores ($\chi^2(3) = 6.39$, $P = 0.0939$), or aseptate hyphae ($\chi^2(3) = 7.01$, $P = 0.0715$); marginally significant for hyaline hyphae ($\chi^2(3) = 7.67$, $P = 0.0533$); and strongly predictive of the incidence of dematiaceous hyphae ($\chi^2(3) = 15.907$, $P = 0.0012$), which declined with depth.

Community composition did not vary with respect to inundation. — We cultured 83 morphologically distinct fungi from 108 plants (Fig. 3). The composition of these communities did not differ with respect to inundation gradients ($F = 1.00$, $P = 0.4750$. Ordination via nonmetric multidimensional scaling suggested a 4-dimensional solution (final stress= 19.67, final instability = 0.0003 over 500 iterations), with four principal axes explaining r^2=0.55 of the variance (Axis 1, r^2= 0.16; Axis 2, r^2= 0.16; Axis 3, r^2= 0.13; Axis 4, r^2=0.09). Samples from inundated transect points are indistinguishable from those in subaerially-exposed soils (Figs. 3A–B).

Fungal incidence and community composition did not vary geographically.. — The incidence of fungi within plant roots did not differ significantly between geographically distant reservoirs (aseptate: $\chi^2(1) = 0$, $P = 0.999$; hyaline: $\chi^2(1) = 0.3$, $P = 0.725$; dematiaceous: $\chi^2(1) = 3.04$, $P = 0.1498$; vesicles: $\chi^2(1) = 0$, $P = 1.0$; spores: $\chi^2(1) = 0.12$, $P = 0.8367$; chytrids: $\chi^2(1) = 0$; $P = 0.9995$). Community composition of culturable fungi (Fig. 3C) was also similar between reservoirs ($F = 1.23$, $P = 0.159$).

DISCUSSION

Our study presents the first explicit investigation of inundation effects on the broader community of root endogenous fungi in wetland plants, expanding upon a previous body of literature focussed on the ecology of arbuscular mycorrhizal fungi (AMF) in wetlands. Previous studies have hypothesized that much as plant tolerance to water depth and sediment anoxia structure aquatic plant communities (Spence 1982, Brix et al. 1992, Lemoine et al. 2012), above-ground zonation in plant adaptation may be mirrored by below-ground zonation of commensal fungi (Anderson et al. 1994, Khan and Belik 1995, Miller and Bever 1999). Our study demonstrates that a variety of root-endogenous fungi exhibit reduced incidence when host roots are inundated, results which are similar to reports of AMF response to inundation. Investigations employing hydrologic gradients (Anderson et al. 1984, Stevens and Peterson 1996, Miller and Bever 1999), or inferring hydrologic effects by sampling different basins with varying depths or soil moisture (Wetzel and van der Valk 1996, Bauer et al. 2003), typically have demonstrated that occurrence and intensity of AMF colonization declines with depth and redox potential (Tanner and Clayton 1985, Miller 2000).

Contrary to our predictions that incidence of root-endogenous fungi would be negatively correlated with inundation depth, reflecting diminishing efficiencies in gas transport into distal root tissues, we found that any degree of inundation diminished the incidence of hyphae compared to roots taken from subaerially-exposed soils. Deeply inundated roots contained similar amounts of hyphae as shallowly-inundated specimens. These trends were apparent for all vegetative mycelia we examined, which comprised simple-septate hyaline hyphae consistent with ascomycete pathogens, endophytes, and/or saprotrophs; dematiaceous, septate hyphae attributable to dark septate endophytes; and aseptate or coenocytic hyphae, which may represent AMF, but could be attributed to other mucoralean fungi (Field et al. 2016). AMF form

associations with *Typha* (Stenlund and Charvat 1994, Wetzel and van der Valk 1996, Turner et al. 2000, Bauer et al. 2003, Dunham et al. 2003, Ray and Inouye 2006), but this may be a facultative rather than obligate mutualism (Dunham et al. 2003, Janos 2007), as many studies also report absence of AMF in *Typha* roots (Anderson et al. 1984, Cornwell et al. 2001). Because vesicles were rare in our *Typha* samples, but occasionally were observed in the co-occurring grass roots, we hold it more likely that the aseptate hyphae in *Typha* roots represent other mucoromycotan fungi, but cannot rule out the possibility that they are glomalean.

The apparent suppression of hyphae that we observed could conceivably result from changes in community composition, with exclusion of obligate aerobes and competitive release of facultative anaerobic fungi. Our results suggest otherwise: we observed exemplars of all three categories of hyphae in roots taken at all depths; the incidence of chytrids, which are facultative anaerobes, did not vary with respect to inundation; and there were no significant differences between communities of cultured fungi, when assessed on the basis of morphotypes. Our culture assays differed from results of assays performed by Sandberg et al. (2014), who demonstrated that although fungal endophyte community diversity was not significantly different between collection periods, among host plants, as a function of depth, or at regional scales (i.e., within *vs*. between watersheds), endophyte community structure could vary significantly between individual reservoirs. Identifying fungi on the basis of colony morphotypes has the disadvantage of 'lumping' genetically-diverse isolates, and probably underestimates species diversity. Nevertheless, our observations of mycelial incidence in roots, taken in conjunction with culture assays, suggest that reduced mycelial incidence is suppression of root-endogenous fungi, and not simply exclusion of fast-growing obligate aerobes that might be expected to predominate in subaerially-exposed soils.

Previous studies of AMF in wetland plants may offer insight into the trends we observed in the broader community of root endogenous fungi in *Typha* plants. We hypothesize that low hyphal incidence in inundated *Typha* roots reflects germinal inhibition or unsuccessful initial colonization, as has been suggested for AMF (Daniels and Trappe, 1980, Le Tacon et al. 1983, Saif 1981, 1983). For AMF, initial colonization is strongly suppressed by inundation (Miller 2000, Miller and Sharitz 2000), but mycorrhizal associations established during dry seasons do not appear to be affected by periodic inundation (Miller and Sharitz 2000, Ray and Inouye 2006, but see Ipsilantis and Sylvia 2007). AMF spores (Khan and Belik 1995, Miller and Bever 1999, Miller 2000, Miller and Sharitz 2000) and asexual spores of other fungi are abundant in wetland soils (Card and Quideau 2010), and frequently concentrated in the wettest portions of hydrologic gradients (Khan and Belik 1995, Miller and Bever 1999) where they may remain viable for many years (Wolfe et al. 2007). Spore germination in inundated soils may be effected by extra-radicle oxygen leakage; indeed, *Typha* stands have been shown capable of oxidizing the entirety of the rhizosphere (Aldridge and Ganf 2003). High redox potential in wetland soils is, however, a condition subject to diel fluctuation: plants transport oxygen to their roots only while photosynthesizing, and surrounding sediments thus become anoxic and reducing at night (Sorrell and Dromgoole 1989, Caffrey and Kemp 1991), while residual pore-water oxygen is consumed by bacteria (Jespersen et al. 1998, Vepraskas and Faulkner 2001, Nikolausz et al. 2008). Fungal spores germinating in the sediments around inundated roots would thus have a narrow temporal window for successful infection of the root environment.

Future research should investigate whether trends identified here hold for root-endogenous fungi in other wetland plants, across a wider variety of hydrological regimes. Mechanistically, pot experiments which address root colonization with a view to redox

conditions would be of great utility in determining whether apparent suppression of root-endogenous fungi results from germinal inhibition, or depressed mycelial proliferation. As we continue to develop a comprehensive understanding of plant-fungal interactions in biologically hostile settings, it is evidently necessary to consider not only the root environments that fungi inhabit, but also the extrinsic biogeochemical factors which may have broad impacts on fungal recruitment and colonization thereof.

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AUTHOR CONTRIBUTIONS

Research was conducted by AAK; the manuscript was written by AAK and BAS.

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LEGENDS

Figure 1. Experimental design. Three transects were established at each reservoir, and rhizomes with attached roots of three plants were taken from every sampling point: deepest- and highest-growing *Typha* plants, the measured median of each transect, and grasses growing adjacent to the highest-growing *Typha* plants (n = 108).

Figure 2. Incidence of fungal structures in plant roots. A. Aseptate hyphae, B. hyaline hyphae, C. dematiaceous hyphae, D. vesicles, E., asexual spores (=conidia/conidiospores), F. epi- and

endo-biontic chytrid sporangia. *X*-axes reflect transect points, as per FIG. 1, lower *y*-axes express the percent of root intersects (as per McGonigle et al. 1990) that contain fungal structures of interest. Shading of bar graphs reflects reservoir identity, as detailed in FIG 2A. Error bars represent the standard error (SE) of the mean. Upper *y*-axes convey the results of Tukey post-hoc multiple pairwise comparisons ($P = <0.05$) for general linear mixed models (GLMM) with negative binomial distributions.

Figure 3. Nonmetric multidimensional scaling plot of $n = 108$ samples, scaled by $n = 83$ visibly distinguishable root-endogenous fungal morphotypes cultured from surface-sterilized *Typha* roots. A., B., samples grouped by inundation, C. samples grouped by reservoir of origin. NMDS analyses indicate a 4-dimensional solution ($r^2_{total} = 0.548$).

FOOTNOTES

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Figure

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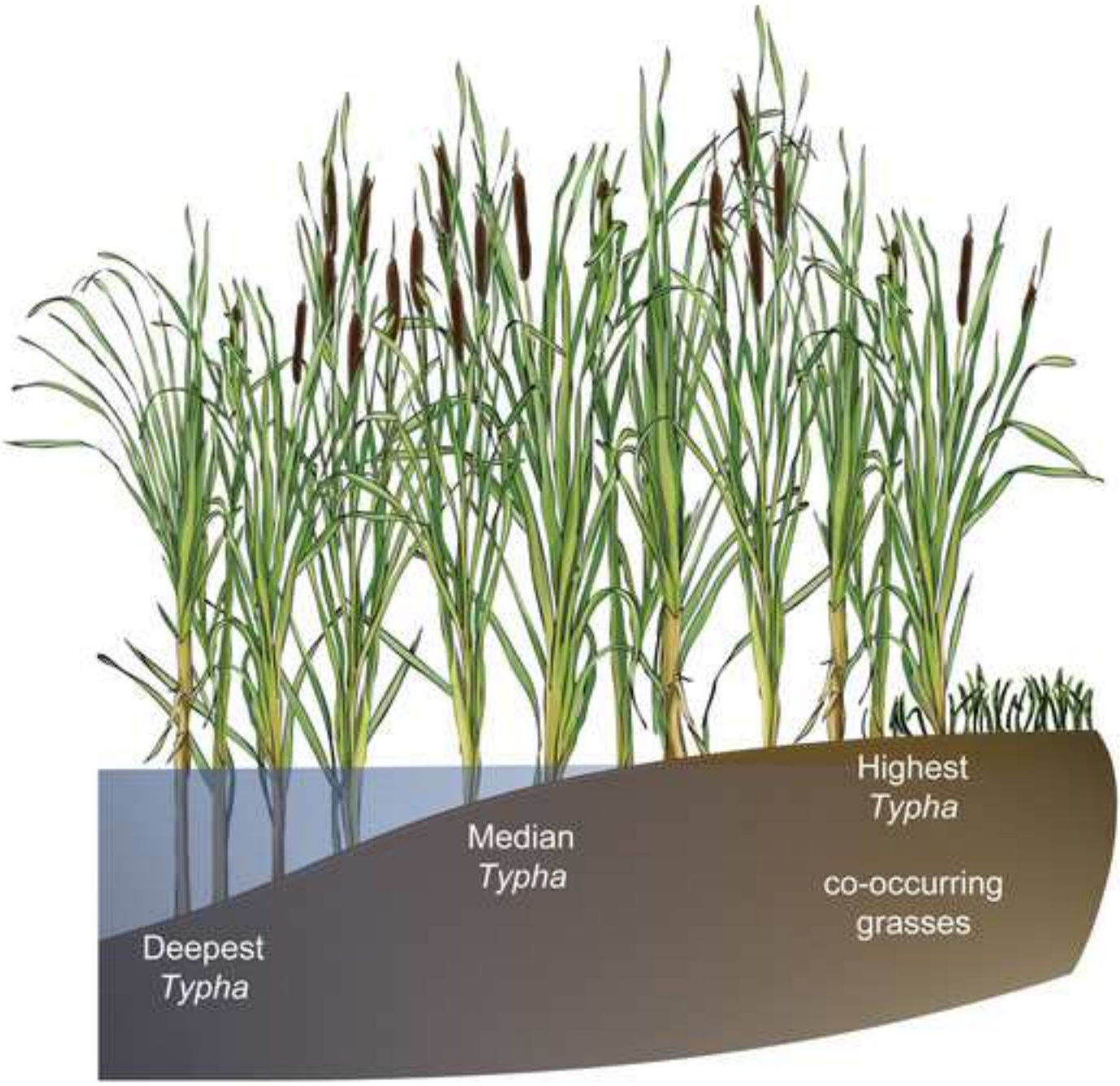

Figure

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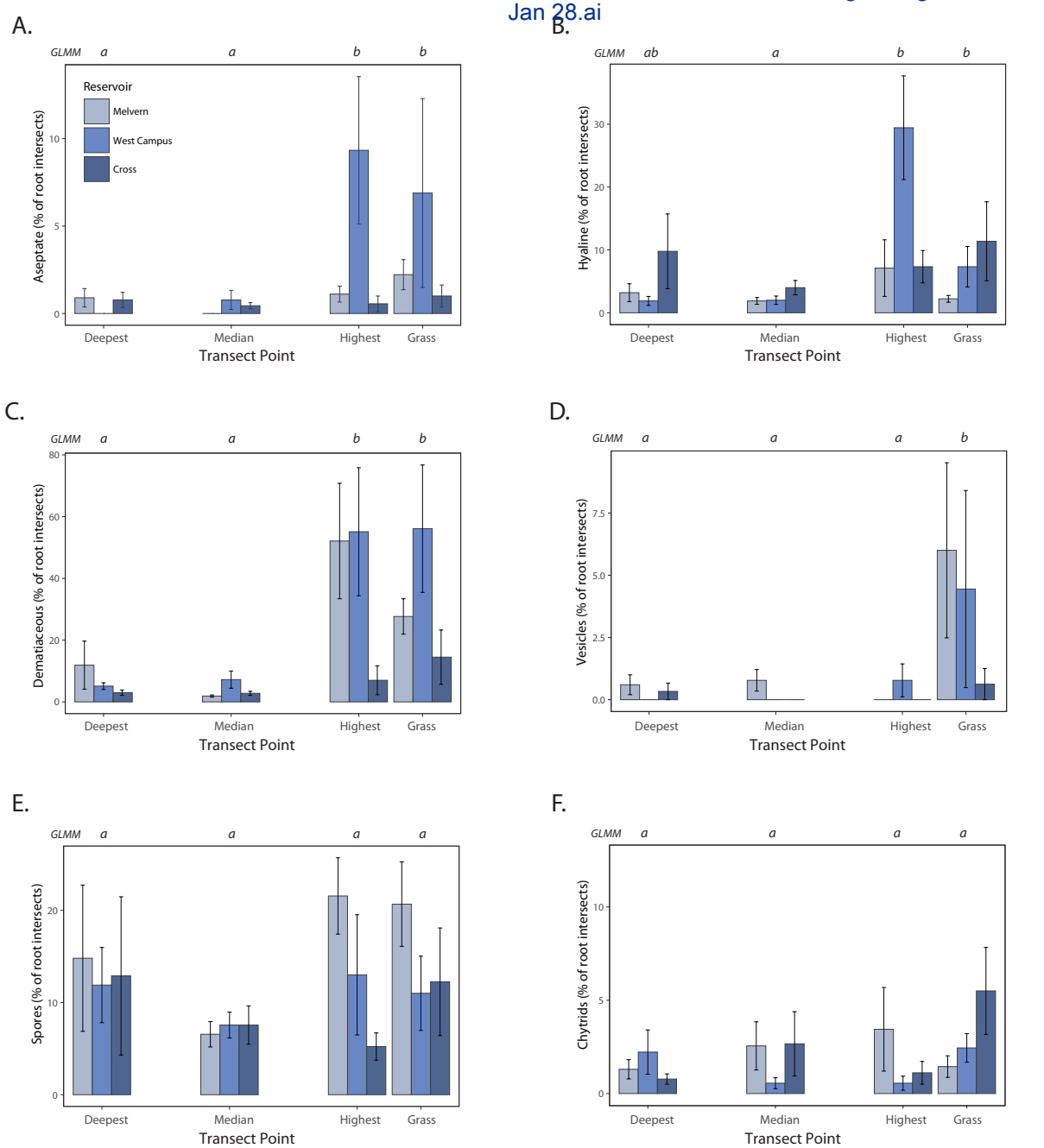

Figure

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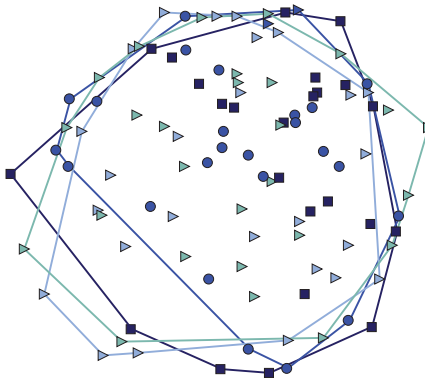
A.

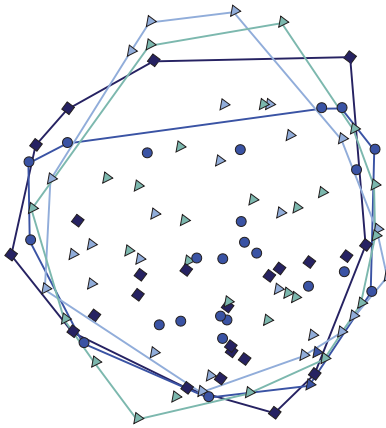
Inundation (transect point)



B.

Inundation rotated by Reservoir

Deepest *Typha*
Median *Typha*
Highest *Typha*
Co-occuring grass



C.

Reservoir

Melvern
West Campus
Cross