ENVIRONMENTAL SEX DETERMINATION IN THE GENUS EQUISETUM: SUGARS INDUCE MALE SEX EXPRESSION IN CULTURED GAMETOPHYTES

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Horsetails (Equisetum, Sphenophyta) are homosporous, and sexual differentiation of Equisetum gametophytes is under the influence of environmental conditions. Still, the environmental cues responsible for sex determination of Equisetum gametophytes in vitro and in the wild have remained elusive. Here, we show that significantly different sex ratios are obtained when gametophytes are grown on media with or without sugar. In our experimental conditions, male gametophytes outnumber females in the presence of 60–120 mM sucrose and 120 mM glucose, whereas in the absence of sugar, most gametophytes differentiate as female. A similar effect is also observed on already differentiated female gametophytes, which become hermaphroditic sooner when cultured in the presence of sucrose in vitro. Interestingly, these results are reproducible within and across species representative of the two subgenera Equisetum and Hippochaete, indicating that the entire genus may share an identical pattern of environmental sex determination.

Keywords: sex determination, Equisetum, environment, gametophyte, in vitro culture, sugars.

Introduction

Seedless vascular plants are a paraphyletic group of plants characterized by a haplo-diplophasic life cycle. Among these, Isoetaceae, Selaginellaceae, and Marsileaceae are heterosporous, i.e., producing micro- and macrospores that differ in morphology, whereas club mosses (Lycopodium), horsetails (Equisetum), and most ferns (Filicophyta) are homosporous. In Equisetum, perennial sporophytes (2n) produce sexually undifferentiated spores (n) that are dispersed and germinate to form photosynthetic male, female, or sometimes bisexual gametophytes (n). Fertilizations between male and female gametophytes (n) result in the formation of new sporophytes.

The sexual determination of Equisetum gametophytes has been problematic for many years, with various authors emphasizing genetic sex determination, environmental sex determination, or a mixture of both (Mohan Ram and Chatterjee 1970; Laroche et al. 1972, 1978; Hauke 1977). However, the observations that (i) archegonial (female) gametophytes always change to antheridial (male) lobe production when cultured over a prolonged time, (ii) secondary archegonial branches can be regenerated from otherwise purely male gametophytes, and (iii) the proportions of male and female gametophytes developing from the same initial set of spores are highly sensitive to growth conditions all indicate that the spores of Equisetum are not individually predetermined for the expression of one sex or the other (Duckett 1970, 1972, 1977, 1979). Instead, in each developing gametophyte, a series of threshold conditions appears to determine which sex is expressed (Duckett 1977).

To better understand sex determination in Equisetum gametophytes, several authors have studied their response to a number of culture conditions in vitro (Mohan Ram and Chatterjee 1970; Hauke 1971, 1977). Studied environmental factors include light quality and level, crowding, pH, and sucrose concentration in the culture media. Although each of these factors seems able to affect the sex ratio, in many cases, the effects are not consistent among different species. For example, sucrose enrichment has been reported (i) to inhibit female sex differentiation in Equisetum ramosissimum (Mohan Ram and Chatterjee 1970), (ii) to decrease the proportion of males in Equisetum arvense (Hauke 1971, 1977), (iii) to increase the proportion of males in E. ramosissimum (Mohan Ram and Chatterjee 1970) and Equisetum byemale (Hauke 1971), or (iv) to have no effect on sex expression in the same E. hyemale (Hauke 1977). Hauke (1977) concluded: “A number of experiments utilizing sugar enrichment were attempted, but the results were so inconsistent that no conclusions can be drawn from them” (p. 29). Such discrepancies may be explained by variations in culture conditions between experiments, leading to the interaction of other important factors such as light quality and crowding. Alternatively, there may be variability among spores collected from different individuals of the same species and/or from individuals of different species.

In an attempt to characterize the pattern of sex expression in Equisetum gametophytes from different species, we conducted repeated and controlled culture experiments in the presence of various sugar concentrations. Also, we examined the stages during which sex differentiation can be modified by sugar. Here, we present evidence that the male-promoting ef-
fect of sucrose and glucose in the culture medium is significant and can be reproduced within and across species of *Equisetum*. We also show that the addition of sucrose to the culture medium can induce mature female gametophytes to change sex. The significance of this finding is discussed in relation to life history in this ancestral lineage of plants and in the light of evolutionary theories of sex determination.

**Material and Methods**

**Spore Collection and Storage**

Spores were collected from one cone per plant. For each *Equisetum* species, plants from two distinct localities were used as sources of spores. *Equisetum palustre* L.: P11, P12, Campus University Paris XI, Orsay, France; P2, northern bank of the Great Pond, Saint-Mars-La-Brière, France. *Equisetum arvense* L.: A1, Campus University Paris XI, Orsay, France; A2, Campus University Paris XI, Bures-sur-Yvette, France. *Equisetum telmateia* Ehr.: T1, Campus University Paris XI, Orsay, France; T2, Moulin de la Mare, Milon-La-Chapelle, France. *Equisetum variegatum* Schleich.: plants cultivated in the Conservatoire National Botanique du Bassin Parisien and originating from (V1) Forest of Marly, Marly, France, and (V2) Botanical garden, Samoens, France.

For *E. variegatum* and *E. palustre*, spores were collected from mature cones and stored in hermetically closed tubes at −70°C. For *E. arvense* and *E. telmateia*, mature but unopened cones were harvested and sterilized for 5 min in 10 g L⁻¹ of calcium hypochlorite to which 50 µL of Teeopol (Lever) detergent per 100 mL was added. Cones were rinsed in distilled water, allowed to dry overnight, and then dissected to release the spores, which were stored as above.

**In Vitro Culture**

G0 culture medium contained the macronutrients of Estelle and Sommerville (1987): 2.5 mM KH₂PO₄ replaced by 2 mM KH₂PO₄ and 0.5 mM K₂HPO₄ so that the medium was directly buffered to pH 6.1, the mineral micronutrients of Murashige and Skoog (1962) diluted by half, and 10 mM Fe EDTA. Phytagel (0.2% w/v; Sigma) and agar (0.4% w/v; Biofit, Fisher) were used as gelling agents before autoclaving for 12 min at 120°C.

Whereas G0 contained no additional component, 120 mM glucose was added to G120G, 30 mM sucrose was added to G30S, 60 mM sucrose was added to G60S, 90 mM sucrose was added to G90S, and 120 mM sucrose was added to G120S. Sixty mM mannitol was added to G60ML for a control as an unmetabolized osmotic agent. Sugars or mannitol were added to the culture medium before sterilization: sucrose and glucose (Prolabo, RP NORMAPUR) and mannitol (Merck).

Gametophyte cultures were placed in a growth chamber at constant 24° ± 1°C and 24 ± 3 mmol m⁻² s⁻¹ of photosynthetically active radiation for 16 h d⁻¹ provided by fluorescent lamps, a 1/1 mixture of Philips TLD36W82, 2700°K, and TLD36W33, 4000°K.

For the primary sexual differentiation experiment, at t = −10 d, spores were sown on G0 petri dishes and then placed in the growth chamber. At t = 0, gametophytes were transferred to test-medium dishes. Test media were G0, G30S, G60S, G90S, G120S, G120G, and G60ML. Sixteen randomly chosen gametophytes were transferred to each test-medium dish, with each medium replicated in three dishes (for P11 and V1) or four dishes (for P2, V2, A1, A2, T1, T2). The gametophytes were observed under a binocular dissecting microscope every week after transfer to the test media until the end of the experiment. For each gametophyte, viability and gender were determined according to the presence or absence of antheridia and archegonia, with each gametophyte recorded as female, male, bisexual, undifferentiated, or dead. At the end of the experiment, 5 wk for *E. telmateia*, 8 wk for *E. arvense* and *E. variegatum*, and 9 wk for *E. palustre*, pairs of gametophytes were transferred into a flooded G30S tube to assess the fertility of some of the males and females grown on G0, G30S, G90S, and G120S. In all cultures, a sporophyte developed after 3–4 wk, indicating that the sex organs and gametes were functional.

For hermaphroditization experiments, spores were sown on G0 dishes, and gametophytes were transferred to G0 for primary sex expression. When the gender of gametophytes could be determined, female gametophytes were transferred to test media and further grown as above. Nine randomly chosen female gametophytes for P12 and 12 female gametophytes for T2 and V1 were transferred to each test-medium dish, and each medium was replicated over three dishes. The female gametophytes were observed every week after transfer to the test-medium plates, and the appearance of antheridia was recorded.

**Statistical Analyses**

The statistical analyses included only plates in which less than three gametophytes failed to grow or to differentiate (undifferentiated + dead < 3). In the early sex-differentiation experiment, the sex ratio was defined as (males + bisexuals)/(males + bisexuals + females), accounting for the observation of male sex expression both in initially male gametophytes and in rare bisexuals deriving from initially female gametophytes. The sex ratio on each plate was square root– and arcsine-transformed and then submitted to ANOVA, with test medium and plant species as fixed effects and plant origin (nested within species) as a random effect (general linear models procedure; SAS Institute 1988). In the hermaphroditization experiment, where only one plant per species was used, the statistical treatment was the same as above, except that the sex ratio on each plate was defined as bisexuals/(bisexuals + females) and that the only effect included in the model was test medium. Tukey’s studentized range test was performed for multiple comparisons. The relation between sucrose concentration and the sex ratio obtained on each dish was assessed by using the SAS regression procedure.

**Results**

To test the effect of sugar addition on sex expression in four species of *Equisetum*, we performed two different experiments, examining the influence of growth medium on sex expression, starting with either 11-d-old undifferentiated *Equisetum* gametophytes (table 1; fig. 1) or older differentiated female *Equisetum* gametophytes (fig. 2).
In the first experiment, the addition of sugar to the culture medium had a highly significant effect on the observed sex ratio \((P < 0.0001; \text{table } 1)\). The species and the origin of plants within each species were also found to be important \((P = 0.0039 \text{ and } P = 0.0079, \text{respectively})\). Plants from a same species showed different responses to the test media \((P < 0.0001)\). However, no significant interaction was found between test-medium effect and species effect \((P = 0.12)\), indicating that the general pattern of sex expression in response to sugar addition is conserved across the four studied *Equisetum* species.

For each plant, additional sucrose and glucose favored the expression of male gender, whereas mannitol never had a significant effect, compared with the G0 mineral medium (fig. 1). The sex ratio per plate increased with increasing sucrose concentration up to 120 mM, with all linear regressions significant \((P < 0.05)\) with positive slopes. For T2 and V2 plants, 120 mM concentration gave significantly higher sex ratios than both 60 mM sucrose (same number of atom gram of carbon) and 120 mM sucrose.

The results showed that sex ratios with an excess of males are obtained when young gametophytes are grown on media with added sugar. The pattern of sex expression described here is in agreement with the now widely held opinion that *Equisetum* spores and gametophytes are potentially bisexual (Duckett 1977). Clearly, the sex ratios obtained on a given growth medium differ when spores from different origins or species are compared (fig. 1). This probably results from genetic differences between genotypes and species. The male-promoting effect of sugar addition is significant and conserved across four species representative of the two subgenera *Equisetum* (*Equisetum arvense*, *Equisetum palustre*, and *Equisetum telmateia*) and *Hippochoaete* (*Equisetum variegatum*). It is not known whether each of the two subgenera is monophyletic, but if this were the case, it would indicate that the observed pattern of environmental sex determination in response to sugar addition would be general for most of the 16 *Equisetum* species. In addition, the result that sucrose addition promotes the expression of male sex in both undifferentiated and mature female gametophytes indicates that a same mechanism of sex determination could operate at these two developmental stages.

To our knowledge, this is the first time that a same change in culture conditions is reported to consistently modify the sex ratio of *Equisetum* gametophytes from different species. In our work, plants from two distinct localities were used as sources of spores for each of four *Equisetum* species. Treatments were replicated in order to allow statistical analysis, and we used a rather stringent criterion (dead + undifferentiated < 3) to minimize sex-biased mortality or inhibition as a possible cause of variation in the analyzed sex ratios. The absence of such care, as well as differences in culture conditions, such as light level, light quality, and basic growth medium (Mohan Ram and Chatterjee 1970; Hauke 1971, 1977), may account for the discrepancies found across previous results. In addition, the statistical analyses used in some of these investigations might have confounded the growth-medium effect with the effect of crowding, a factor that itself seems able to modify the sex ratio of cultured *Equisetum* gametophytes (Hauke 1977).

We conclude that sugars act as nutrients in our experiments because they enhance the growth rate of cultured gametophytes. However, the effect of sugar on *Equisetum* sex ratios is contrary to the effect of light; in fact, the sex ratios are more female biased with increasing light levels (J.-M. Guillon, unpublished data). Hauke (1971) already concluded that the morphogenetic effect of sucrose on *Equisetum* gametophytes was independent of any nutritional effect but, rather, superimposed on the morphogenetic effects of light. Two hypotheses emerge from this picture: (i) the male-promoting effect of light is not exclusively mediated by the photosynthesized carbohydrates, and/or (ii) the addition of exogenous sugars has other physiological effects besides mimicking the photosynthesis of carbohydrates. Concerning the second proposition, it is possible that the presence of exogenous sugars is a stress to the young developing gametophyte. Specifically, osmotic

### Table 1

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**Discussion**

**Sugar Addition Favors Maleness in Cultured Equisetum Gametophytes**

**Table 1**

ANOVA of Test-Medium Effect, Plant Origin Effect (Nested within Species), Plant Species Effect, and All Possible Two-Way Interactions on the Sex Ratios Observed in the Early Sex Differentiation Experiment (Fig. 1)
Fig. 1 Sex ratios in cultures of *Equisetum* gametophytes. For each plant, bars represent the mean sex ratio ([male + bisexual]/[male + bisexual + female]) and associated standard error obtained on the corresponding test medium. The number of repeats (n) and the result of multiple comparisons (Tukey’s studentized range test) are also indicated. Treatments with the same letter do not significantly differ (P > 0.05). Treatments that differ from G0 (P < 0.05) are indicated with bars in black. The duration of growth on test media is specified with the name of the plant. A1 and A2, *Equisetum arvense*; P11 and P2, *Equisetum palustre*; T1 and T2, *Equisetum telmateia*; V1 and V2, *Equisetum variegatum*.

When reviewing the variations in sex ratio of cultured *Equisetum* gametophytes, investigators have noticed that under conditions favoring vigorous vegetative growth of young gametophytes, e.g., high light levels, most spores differentiate into initially female individuals (Duckett 1970). However, less...
Fig. 2 Timing of hermaphrodite appearance in initially female gametophyte cultures of P12 (Equisetum palustre), T2 (Equisetum telmateia), and V1 (Equisetum variegatum). For each plant and test medium, the mean sex ratio with its associated standard error is shown as a function of time. Each treatment was replicated in three dishes. Circles, G0 (no sucrose added); squares, G60S (60 mM sucrose); triangles, G90S (90 mM sucrose).

Evolutionary Significance of Sex Determination in Equisetum Gametophytes

Equisetum species have a haplo-diplophasic life cycle, characterized by the alternation of a perennial sporophytic generation and a short-lived gametophytic generation. Not all sporophyte populations produce spores, and they can propagate efficiently through vegetative reproduction. New populations may be founded either by broken-off or dispersed vegetative parts of the sporophytes or by dispersed spores and sexual reproduction between emerging gametophytes. Studies of genetic diversity in E. arvense and Equisetum hyemale populations, as well as high fertilization rates found in wild gametophyte populations, indicate that sexual reproduction occurs and cannot be dismissed as marginal in Equisetum (Duckett and Duckett 1980; Soltis et al. 1988; Korpelainen and Kolkkala 1996).

In the wild, observed differences in sex ratios between populations of the same species at the same time and between populations at the same locality during different years all indicate an important environmental component for sex determination in Equisetum (Duckett and Duckett 1980). Evolutionary theories predict that environmental sex determination is favored (i) when early in life, an individual enters a patchy environment that has a large effect on its lifetime fitness (some patches being relatively more beneficial to females than to males and some being the opposite), (ii) when individuals have little control over which patch they enter, and (iii) when individuals from different patches mate randomly (Charnov and Bull 1977; Bull 1981). Equisetum gametophytes might fit this model if natural populations inhabited patchy places made of microenvironments conferring more or less an advantage to one sex. It has also been shown that short-lived species are less prone to evolve environmental sex determination because environmental fluctuations may cause detrimental sex-ratio variations in such a system (Bull and Bulmer 1989). For this reason, strong sex-specific fitness effects of the environment would be expected in order to explain the occurrence of environmental sex determination in the seasonal Equisetum gametophytes in the wild. Since the literature does not provide such fitness-related data, new work is clearly needed to examine whether the biology of Equisetum gametophytes conforms to the predictions of the Charnov and Bull (1977) model.

As shown here, male sex expression can be induced in four species of Equisetum by the addition of sugar to the growth medium. However, the addition of sugar in our experimental design does not mimic any specific environmental factor that gametophytes would encounter in the wild. Hence, sex determination in response to sugar concentration is unlikely to have evolved per se, but exogenous sugars must somehow interfere with the physiology of the developing gametophyte in such a way that sex determination is affected. Sex determination in horsetails seems to integrate many physiological signals arising from the interaction of the gametophyte with its environment. Indeed, many environmental factors, such as light quality and level, competition, and pH, are also able to modify sex expression in Equisetum gametophytes (Mohan Ram and Chatterjee 1970; Hauke 1971, 1977). In this context, it is quite understandable that sugar concentration in the growth medium is one among many factors that influence sex determination of gametophytes cultured in vitro.

Lability of sex expression occurs in many plant taxa, but only in homosporous pteridophytes is lable sex the rule (Korpelainen 1998). Indeed, sex determination in horsetails is reminiscent of that found in ferns, where undifferentiated gene-
tophytes respond to antheridogen hormones secreted by female gametophytes. The evolutionary significance of this information system in fern gametophytes is still a matter of debate (Willson 1981; Haig and Westoby 1988; Korpelainen 1998). Because ferns and horsetails are sister clades (Kenrick and Crane 1997; Pryer et al. 2001), sex determination in horsetails could be evolutionarily related to that found in ferns, but antheridogen hormones have not been detected in horsetails (Hauke 1971). Alternatively, environmental sex determination might have evolved more than once in relation to the distinctive life history of ferns and horsetails because they both are homosporous haplo-diplophasic land plants with perennial sporophytes and autonomous short-lived gametophytes. In particular, it has been proposed that the extreme lability in the sex expression of homosporous pteridophytes may be primarily related to their peculiar mating system (Korpelainen 1998).

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