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The sex of the mother plant affects the sex variation in the offspring in *Dioscorea rotundata* (Poir.)

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ABSTRACT

In cultivated yams *Dioscorea rotundata*, spontaneous sex variation occurs in the offspring despite vegetative propagation. In this study, sex variation was investigated as a function of the region (head, middle, or base) of the seed-tuber and the sex of the mother plant. Tuber-seeds were sown from the head, middle, and base regions of tubers of 6 cultivars, and germination, flowering and sex of the offspring were studied. To determine a possible relationship between the hormone content of the mother tuber and sex variation, the phytohormone profile of the head, middle, and base regions was compared in tubers from male, female, and monoecious plants. Our results showed that the region of the seed-tuber affected germination, as the germination rate of seed-tubers from the head region was 20–50 % higher than that of the middle and base regions, and they germinated 10 days earlier. The region of the seed-tuber also affected the flowering rate, which was 10–20 % higher for seed-tubers from the head region than from the base region, but it did not affect the sex of the offspring. Sex variations in the offspring were more related to the sex of the parents. Male and female cultivars had stable offspring, while monoecious cultivars had high sex variation (80 %) in their offspring. The hormonal profile of the tubers suggested a relationship between hormones and sex identity. Jasmonates and salicylic acid were more concentrated in the tubers of male and monoecious plants, while abscisic acid types, cytokinins and benzoic acid were more concentrated in the tubers of female plants. Further work is needed to clarify the role of these hormones in the control of sex expression in cultivated yam *D. rotundata*.

1. Introduction

Yam is one of the main crops in the tropics and subtropics. The genus *Dioscorea* contains >630 species (Sugihara et al., 2021), but only a few are cultivated. Cultivated yam species vary from region to region, but *D. rotundata* (Poir.) (syn. *Dioscorea cayenensis-rotundata* (L.)) is widely cultivated in West Africa (Price et al., 2018) and particularly in Benin (Dansi et al., 1999). In Benin, it is cultivated in the centre and north of the country where it is the staple food of the population (Aboudou and Auriole, 2006). Yam production has almost doubled in the last decade thanks to new agricultural technologies, an increase in cultivated area, and high demand due to population growth (FAOSTAT, 2024). However, yields remain 5 times lower than expected (FAOSTAT, 2024). Phytosanitary problems, droughts or floods related to climate change (Mani et al., 2016; Ignatius et al., 2019; Ntui et al., 2021) are all causes of these low yields. In addition, little research has been done on the genetic improvement of cultivated yam *D. rotundata* (Denadi et al., 2022). The use of vegetative reproduction as the main mode of propagation could be one of the reasons for the low genetic improvement. Nevertheless, several studies have shown that flowering and fruiting are conserved in yam, so sexual reproduction could produce flowering plants with tubers (Assaba et al., 2018; Mondo et al., 2022, 2020). *Dioscorea rotundata* is essentially characterised by dioecy, although monoecious plants with male and female flowers on the same plant have been observed (Darkwa et al., 2020).

Even though sexual reproduction offers the possibility of genetic improvement and/or of seed production, vegetative reproduction from tubers remains the only option used by yam producers to date.

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Except for *D. esculenta, D. trifida* and *D. bulbifera*, which are grown from whole tubers (microtubers), the other species of the genus *Dioscorea* are generally propagated from tuber fragments (Mathurin and Degras, 1975; Brice et al., 2016). For *D. rotundata*, growers use small tubers from the second harvest (for early two-harvest cultivars). However, for late one-harvest cultivars or in the absence of a second harvest for the early cultivars, growers use relatively long tubers that are usually cut to generate seed-tuber sections (head, middle and base) or sometimes "minisets" (Brice et al., 2016). The use of these long tubers for propagation is at the expense of tuber yield.

Studies focusing on the characterisation of yam flowering have repeatedly mentioned spontaneous variation in sex, sometimes with a difference in sex between individuals from the same tuber (Dansi et al., 1999; Girma et al., 2019). For example, Dansi et al. (1999) obtained both a female and a male individual from two fragments of the same tuber produced by a female plant. These observations have motivated studies focused on the issue of genetic sex determination in yam in recent years (Tamiru et al., 2017; Alabi et al., 2019; Cormier et al., 2019; Denadi et al., 2020). These studies have shown that sex identity is genetically determined in *D. rotundata,* but that sex determination, like most traits within the species, is also strongly influenced by the environment (Alabi et al., 2019). Sex expression and flowering intensity are also influenced by agronomic practices such as plant pruning, tuber removal and hormone application in this species (Mondo et al., 2023). Spontaneous sex changes due to environmental factors have been observed in other monoecious and dioecious crops such as *Zea mays, Elaeis guineensis, Rumex hastatulus, Humulus japonicus* and *Cannabis sativa* (Freeman et al., 1980; Adam et al., 2011; Orozco-Arroyo et al., 2012). In 1952, Sawada observed in *D. batatas* that fragments from the head of the tuber germinated earlier than fragments from the middle or the base of the tuber (Sawada, 1952). In 1957, Miège observed that starch grains were not evenly distributed along the tuber in *D. cayenensis* and *D. alata* and that this uneven distribution affected the germination of the seed-tuber fragments and the yield of the daughter plants (Miège, 1957). In *D. rotundata*, the effect of the type of seed-tuber (whole tuber or fragment) and its origin (head, middle or base) on germination has also been studied (Kalu et al., 1989; Zoundjihekpon et al., 1995; Ayankanmi et al., 2005; Ondo Ovono et al., 2007;). However, none of these studies have investigated the influence of the seed-tuber on the sex variations that are often observed.

The aim of this study was to evaluate the influence of the seed-tuber fragment (head, middle and base) and the sex of the parent plant on germination, flowering and sex variation in the offspring. We also considered whether a possible phytohormone gradient along a tuber could explain the observed changes. Understanding the factors that influence germination, flowering and sex expression in *D. rotundata* is crucial for the development of cultivation and breeding programs for the species.

2. Materials and methods

2.1. Plant material

Seven of the most widely cultivated cultivars (Laboko, Agatou, Katala, Alakissa, Gnidou, Flou and Anago) of yam *Dioscorea rotundata,* grouped in 3 categories, were used in this study:

- Male (M): Alakissa and Flou; plants of these cultivars usually produce male flowers.
- Female (F): Gnidou and Anago; plants of these cultivars usually produce female flowers.
- Mix (Mx): Agatou, Laboko and Katala; plants of these cultivars produce either male, or female or monoecious plants.

Six of these (Agatou, Katala, Alakissa, Gnidou, Flou and Anago) were used for the field experiments and two (Katala and Laboko) with the tree sexual types (male female and monoecious) were subsequently used for hormone quantification.

2.2. Experimental site

Field trials were carried out at the 'Centre de Recherche, de Formation, d'Incubation et d'Innovation pour le Développement Agricole' (CREFIISDA) in Benin. This centre covers an area of 24 ha and is located in the commune of Zogbodomey (village of Lomey, 6°56′ –7° 08′ North, 1°58′ - 2°24′ East). This region has herbaceous vegetation on clay soils with good water retention, which is favourable for yam cultivation. The average annual temperature is 26.8 °C, the annual rainfall is 1244 mm and the average photoperiod is 12/12 h.

Seed-tuber production, sowing and experimental design

The field trial covered two consecutive years (2019 and 2020). In the first year, the effect of the part of the tuber used as planting material on the germination, flowering and sex identity of the offspring was studied. In the second year, the effect of the sex of the mother plant on the sex identity of the offspring was studied.

At the end of the 2017–2018 agricultural season, mother plants of the different cultivars were selected in the field at full bloom and their sex identity was determined. The selected plants were weaned in December 2018 and their tubers were kept on site for one month before being dug up and transferred to the CREFIISDA attic, where they spent a further 3 months in dormancy. In this way, the origin and sex of the mother plant was known for each tuber.

In the first year of the field trial, seed-tuber production was based on healthy mother tubers with an average fresh weight of 800 to 2000 g. The tubers were cut transversely to obtain three pieces per tuber from the nodal (head), proximal (middle) and distal (base) regions (Fig. 1). These fragments were healed with dry ash for 3 days in the shade. Twenty fragments per tuber region and cultivar were used as seed-tubers (Table S1). For cultivars Alakissa and Flou, all tuber fragments came from male plants; for cultivars Gnidou and Anago, all tuber fragments came from female plants; for cultivars Agatou and Katala, tuber fragments came from 12 monoecious plants, 4 female and 4 male, to match the sex ratio observed naturally in the field (2017–2018 survey). For sowing, each tuber fragment was covered with a mound of soil, with 12 mounds per line, for a total of 36 lines (Figures S1, S2). The mounds were separated by 1.5 m. Sowing took place on 11 May 2019 according to a split-plot design (Figure S1).

In the second year of the field trial, whole tubers from the first year were used as seed-tubers. Between 26 and 32 tubers were used for male (Alakissa, Flou) and female (Gnidou, Anago) cultivars and 37 tubers (8 from males, 18 from females and 11 from monoecious) and

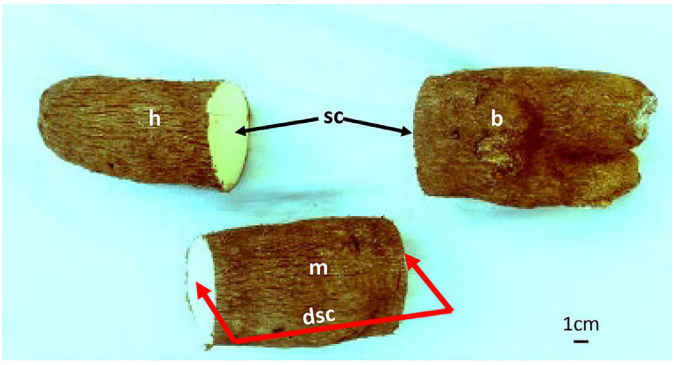

Fig. 1. Seed-tuber production by cutting a fresh whole tuber (30–40 cm; 800–2000 g) into three pieces, the head (h) and base (b) with a single scar (sc) and the middle (m) with a double scar (dsc).

47 tubers (13 from males, 9 from females and 25 from monoecious) were used for Katala and Agatou, respectively (Table S1). Sowing was performed on 15 May 2020 using the same design and method as previously described.

2.3. Measured parameters

In the first year, the germination date, the flowering date and the sex (male, female or monoecious) of each plant were determined. The germination rate (GR, %) was calculated as the ratio between the number of germinated and sown tubers. Flowering rate (FR, %) was calculated as the ratio between the number of flowering and germinated plants. Germination time (GT) was calculated as the number of days between the sowing and the germination dates. Flowering time (FT) was calculated as the number of days between the dates of germination and the flowering. Germination was considered when the plantlet emerged from the soil; flowering was considered when the first inflorescence bud was macroscopically visible. In the second year, only the sex of the plant was determined.

2.4. Hormonal quantification in the tubers by HPLC method

Using the HPLC method, the endogenous phytohormone profile along the tuber was determined in freshly harvested tubers of male, female, and monoecious plants of *D. rotundata* cultivars Katala and Laboko. These cultivars were selected for their ability to produce male, female and monoecious plants. Analyses were carried out in three biological replicates for each cultivar and sex. From each tuber, 100 to 300 mg of fresh material was taken from each region (head, middle, and base); sampling was performed at the centre of each tuber slice. A total of 54 samples were analysed, 27 per cultivar. For ease of reference, the term 'phytohormone' was used in a broad sense to include the classical phytohormones as well as other hormone-like endogenous regulators. In total, six categories of compounds were detected and quantified: (1) cytokinins (CKs): bioactive CKs (trans-zeatin, cis-zeatin, dihydrozeatin, isopentenyl adenine), CKs precursors (trans-zeatin riboside monophosphate, cis-zeatin riboside monophosphate, dihydrozeatin riboside monophosphate, isopentenyl adenosine monophosphate), CKs metabolites (trans-zeatin riboside, trans-zeatin-9-glucoside, cis-zeatin riboside, cis-zeatin-9-glucoside, cis-zeatin riboside-O-glucoside, dihydrozeatin riboside, dihydrozeatin-7-glucoside, dihydrozeatin-9-glucoside, dihydrozeatin riboside-O-glucoside, isopentenyl adenosine, 2-methylthio zeatin riboside); (2) auxins: bioactive auxin (indole-3-acetic acid, IAA), IAA precursor (indole-3-acetamide), IAA metabolites (IAA-Aspartate, oxo-IAA, oxo-IAA-glucose ester), PAA (phenylacetic acid, weak auxin activity); (3) ABA-types: bioactive ABA (abscisic acid), ABA metabolites (dihydrophaseic acid, phaseic acid, ABA-glucose ester, neophaseic acid, 9-hydroxy-ABA); (4) jasmonates: bioactive JA (jasmonic acid), bioactive JA metabolite (JA-isoleucine) and JA precursor (cisOPDA); (5) salicylic acid (SA); (6) benzoïc acid (BzA). Phytohormones were extracted with methanol/formic acid/water (15:1:4, by volume) from tissues frozen and homogenized in liquid nitrogen and then purified by the dual-mode solid-phase method of Dobrev and Kamınek (2002). Two phytohormone fractions were obtained; fraction A (eluted with methanol) containing the acidic and neutral hormones (auxins, SA, BzA, jasmonates, ABA-types) and fraction B (eluted with 0.35 M NH_4OH in 70 % methanol) containing the hormones of basic character (CKs). Hormone analysis and quantification was performed by HPLC (Ultimate 3000, Dionex, Sunnyvale, CA, USA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP; Applied Biosystems, Foster City, CA, USA) set in selected reaction-monitoring mode as described in Dobrev and Vankova (2012). Phytohormones were quantified using a multilevel calibration curve with 2H-labelled internal standards (as described in detail in Dobrev and Vankova (2012) and Djilianov et al. (2013)). The results are presented as the total concentration of CKs, auxins, ABA-types and jasmonates (sum of the concentration of each compound for each phytohormone group). The detailed data for each compound are given in Table S2.

After sampling for hormonal analysis, the tuber parts were left to dry so to allow the cuts to heal and then were sown in 14 cm diameter pots containing a mixture of 1/3 sand and 2/3 potting soil. The pots were placed in a tropical greenhouse in Louvain-la-Neuve (Belgium) (average temperature 29 °C/28 °C (day/night); relative humidity 65 %; photoperiod 16 h; minimum light intensity 180 μmol/m^{2}s) and were watered with rainwater 1 to 3 times per week depending on the external temperature conditions. Unfortunately, no seedlings emerged from the soil (even after several weeks) and many of the seed-tubers rooted, so it was not possible to determine the sex of the offspring.

2.5. Statistical analysis

Statistical analyses were performed using JNP Pro 14 software (version 2019). Logistic regression was used to measure the effect of cultivar, sex and tuber region on germination and flowering rates. A 3-way ANOVA was then performed to measure the effect of cultivar, sex and tuber region and their interactions on germination and flowering times. Linear regression was also used to estimate flowering time from germination. Prior to these analyses, the normality of the residuals was checked using plots (box plots and qq plots) and homogeneity of variances using Levene's test. A Tukey HSD test was performed to compare mean germination and flowering times between tuber regions within the same cultivar and between cultivars within the same region. A logistic model was used to measure the effect of tuber region, flowering time, parental sex and possibly their different interactions on sex variations in the offspring.

For the hormonal profile, a 3-way ANOVA was performed to highlight the effects of region, sex and cultivar and their interactions on hormone concentrations. Prior to the analyses, the normality of the residuals was checked using plots (box plots and qq plots) and the homogeneity of the variances on the basis of Levene's test. The Tukey HSD test was used to compare the hormone concentrations between regions and sexes within the same cultivar. A heatmap of the hormone profile was generated using Rstudio (R.4.4.1 version) and the 'shiny' and 'heatmaply' packages with Euclidean distance, scale transformation and wardD2 clustering linkage as settings.

3. Results

3.1. Effect of tuber region and cultivar on tuber germination

Overall, 54.44 % of the tuber fragments germinated (Fig. 2A). Neither the cultivar (χ^2 = 10.24, P = 0.0685), nor the sex of the mother plant (χ^2 = 0.22, P = 0.8663) affected the germination rate. However, the germination rate varied among tuber sections (χ^2 = 61.29, $P < 0.0001$, Fig. 2A): seed-tubers from the head had a higher germination rate (78 %) than those from the base (56 %) and middle (30 %) of the tuber. The effect of the tuber section depended on the cultivar (χ2 = 24.35, P = 0.0067). The difference in germination rate among tuber regions was indeed significant for the cultivars Flou (χ^2 = 14.79, P = 0.0006), Anago (χ^2 = 17.81, $P < 0.0001$), Gnidou (χ^2 = 10.72, P = 0.0047) and Katala (χ^2 = 27.21, $P < 0.0001$) but not for Alakissa (χ^2 = 0.93, P = 0.6258) and Agatou (χ^2 = 0.55, P = 0.2790). Seed-tubers from the middle of the tuber had a particularly low germination rate (< 20 %) in the Flou, Anago, Gnidou, and Katala cultivars (Fig. 2A).

The germination time varied with cultivar (F = 4.49, P = 0.0003) and tuber section (F = 1.76, $P < 0.0001$) but not with the sex of the mother plant (F = 1.72, P = 0.1807, Fig. 2B). Overall, seeds from the head of the tuber germinated much earlier (28 days) than those from

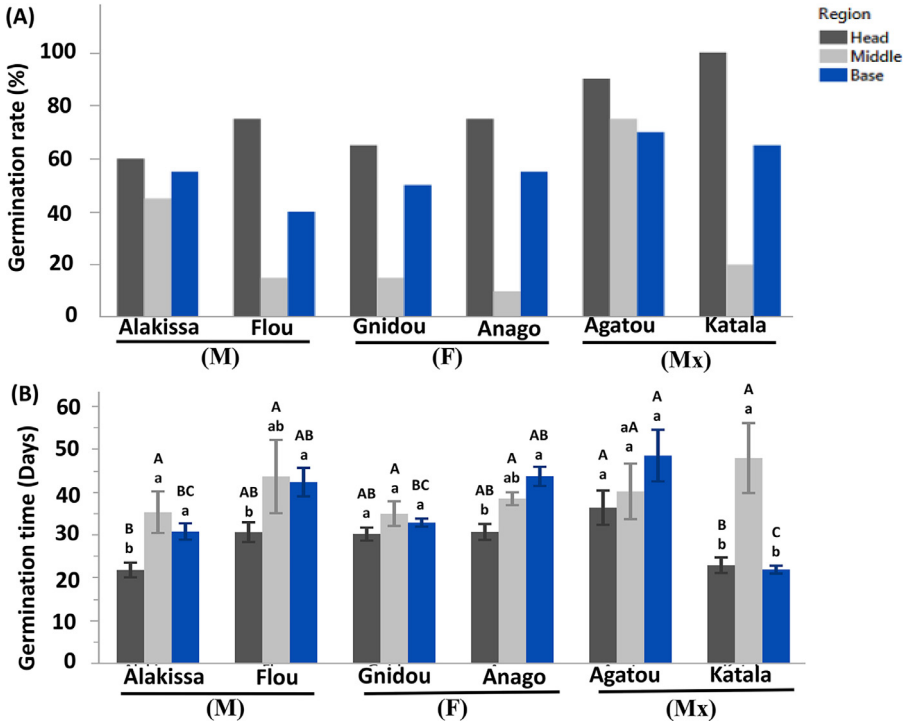

Fig. 2. Impact of the tuber section (head, middle, base) on the tuber germination of six cultivars of *D. rotundata*. (A) Germination rate. (B) Time from sowing to germination. In (B), tuber regions with a same lower case letter for a same cultivar are not significantly different at the 5 % level; cultivars with a same capital letter for a same tuber region are not significantly different at the 5 % level. Data are means ± SE. M: male cultivars, F: female cultivars, Mx: cultivars with male, female or monoecious plants.

the base (36 days) and middle (40 days) of the tuber. However, germination times were relatively similar between tuber regions in the cultivars Gnidou (F = 1.74, P = 0.1970) and Agatou (F = 1.25, P = 0.1949) (Fig. 2B). As for the germination rate, the effect of the tuber section depended on the cultivar (F = 2.79, P = 0.0031). For the head region, the cultivars Alakissa and Katala showed a lower germination time (22 and 23 days respectively) than Agatou (35 days) (Fig. 2B). For the base region, Katala germinated significantly earlier (22 days) than Anago (44 days), Flou (44 days) and Agatou (49 days). For the middle region, no difference in germination time was observedbetween cultivars .

3.2. Effect of tuber region and cultivar on flowering

The flowering rate varied according to the cultivar (χ^2 = 88.34, $P < 0.0001$, Table 1): Alakissa showed the highest flowering rate (94 %) while Anago did not flower. The tuber section also affected the flowering rate (χ^2 = 22.08, $P < 0.0001$, Table 1). The cultivars Agatou and Katala (Mx) showed relatively higher flowering rates in plants from head regions (78 % and 95 % respectively) than in those from basal regions (71 % and 68 % respectively). In contrast, the cultivars Alakissa, Flou (M) and Gnidou (F) had similar flowering rates between tuber regions (χ2 = 4.49, P = 0.1056). It is important to recall that the germination rate of the middle tuber region was low so there are few plants from this region compared to plants from the head and base regions (Table 1). However, in Agatou, where all regions showed a germination rate above 50 %, the flowering rate of plants from the middle tuber region was very low (6.67 %) compared to plants from the head (64.86 %) and base (70.50 %) regions. Parental sex also had a significant effect on flowering rate (χ2 = 34.91, $P < 0.0001$). The flowering rate of male cultivars was higher (92.54 %) compared to monoecious (60.87 %) and female plants (44.26 %). Very few plants from both male and female parents flowered for the cultivars Agatou and Katala.

Flowering time was affected by cultivar (F = 20.84, $P < 0.0001$) but not by tuber region (F = 0.37, P = 0.1575) although the interaction between region and cultivar was significant (F = 7.18, $P < 0.0001$). When cultivars were compared independently of tuber region, Alakissa had a significantly longer flowering time (82 days) than Agatou (68 days) and Flou (62 days) while Gnidou (71 days) flowered at the

Table 1
Impact of the tuber section (head, middle, base) on the flowering parameters of six cultivars of *D. rotundata*.

Region	Head			Middle			Base		
Cultivar	n	flowering		n	flowering		n	flowering	
		rate(%)	time (days)		rate(%)	time (days)		rate (%)	time (days)
Alakissa (M)	12	100	86.67±0.64	9	88.9	79.75±1.78	11	100	79.09±1.49
Flou (M)	15	100	64.60±1.80	3	66.7	59.50±0.5	8	100	60.50±1.00
Gnidou (F)	13	92.3	75±2.08	3	100	76.67±4.41	10	100	81.20±1.68
Anago (F)	11	0	–	2	0	–	11	0	–
Agatou (Mx)	18	77.8	64.86±3.03	15	6.7	81	14	50	70.50±2.92
Katala (Mx)	20	95	74.32±1.8	4	50	64.00±12	13	53.9	67.57±2.69

M: male cultivars, F: female cultivars, Mx: cultivars with male, female or monoecious plants.

same time as Katala (78 days) and Agatou (68 days). Plant sex had no significant effect on flowering time (F = 0.41, P = 0.6601). However, female plants flowered slightly later (78 days) than males (72 days) and monoecious plants (70 days).

Furthermore, the analyses also showed a significant effect of germination time on flowering time (F = 22.94, $P < 0.0001$) such that, for most cultivars, flowering time decreased with increasing germination time (r^2 = 0.70).

3.3. Effect of tuber region and cultivar on sex variation in the offspring

Overall, the sex variation in the offspring did not depend on the tuber section (χ^2 = 0.01, P = 0.9053, Fig. 3A) but rather on the sex of the mother plant (trial 1: χ^2 = 85.951, $P < 0.0001$, trial 2: χ^2 = 107.65, $P < 0.0001$, Fig. 3). All plants of the male cultivars Alakissa and Flou produced male flowers like their parents in both trials; no sex variation was observed in these male cultivars. Similarly, the female cultivars Gnidou and Anago mainly produced female plants like their parents (Fig. 3). The only exception was Gnidou where a monoecious plant was observed in both trials. In contrast, sex variation was

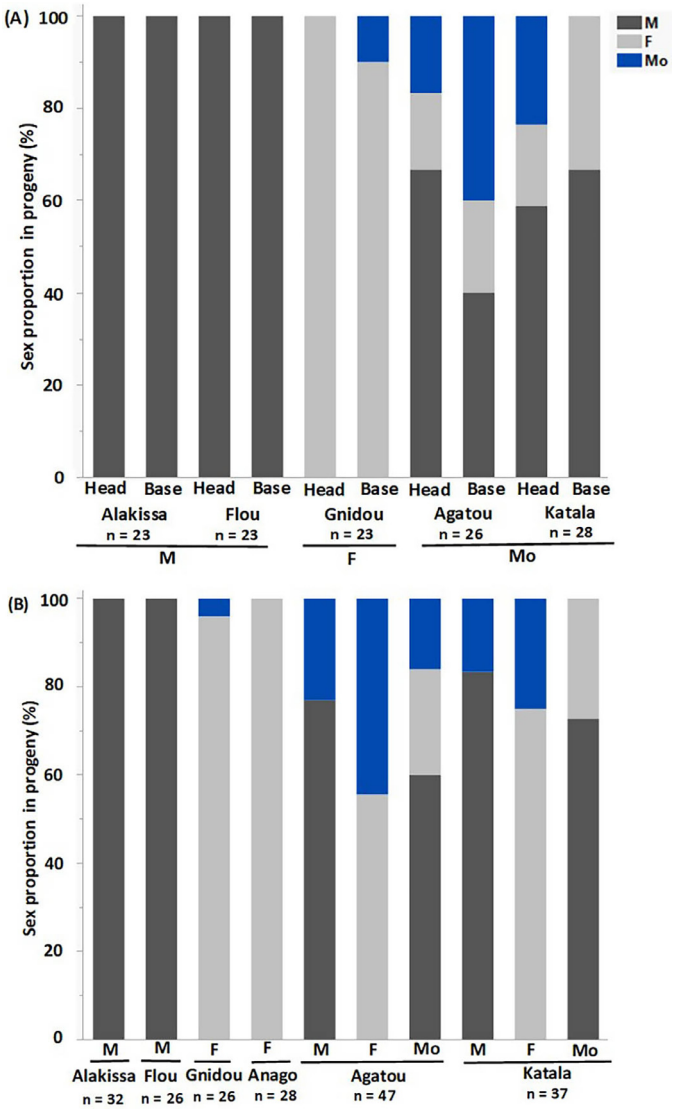

Fig. 3. Sex expression in the offspring as a function of the seed-tuber section and the sex of the mother plants in six cultivars of *D. rotundata*. (A) Results of the first trial comparing the seed-tuber sections (head, base) and the sex of the mother plants. (B) Results of the second trial comparing the sex of the mother plants. M: male plants, F: female plants, Mo: monoecious plants.

important in the monoecious cultivars (Agatou and Katala) irrespective of the seed-tuber section (Fig. 3): both head and base seed-tuber offspring expressed very little of the parental sex. In cultivar Agatou, the percentage of sex variation in the offspring was about 80 % for plants from the head region and 83 % for plants from the base region of the tuber (Fig. 3A). In Katala, these percentages were 76.5 % and 100 % respectively for the head and base regions of the tuber (Fig. 3A). The flowering rate of plants from the middle region was too low to allow comparisons. In the first field trial (Fig. 3A), 75 % of the Agatou and Katala plants that had changed sex produced male flowers and 25 % produced female flowers. In the second field trial (Fig. 3B), 80 % of the monoecious Agatou and Katala plants changed sex in their offspring; 69 % of these produced male flowers and 31 % produced female flowers. We also observed sex variation in male (23 %) and female (44 %) plants of Agatou and Katala, all of which produced monoecious plants in their offspring (Fig. 3B). Thus, the sex variation in the offspring was more related to the sex of the parent plants and the cultivars subject to sex variation were those with mixed flowering (Agatou and Katala). It should also be noted that the observed variation did not depend on the flowering time of the cultivars (χ2 = 0.83, P = 0.3596).

3.4. Phytohormone profile in tubers depending on the sex of the mother plant

In search of intrinsic factors underlying sex variation in the offspring, a phytohormone profile was established in three regions (head, middle and base) of tubers of male, female and monoecious plants. This analysis was performed on two cultivars, Katala and Laboko. About thirty hormones and their metabolites were identified and grouped by hormone type: abscisic acid (ABA) and its derivatives, auxins, salicylic acid (SA), benzoic acid (BzA), jasmonates (JA), and cytokinins (CKs). Full data are provided in Table S2. No ACC (1-aminocyclopropane-1-carboxylate, ethylene precursor) was detected as the concentrations were probably below the threshold value. As shown in Fig. 4, the hormonal profile differed mainly according to the variety and the sex of the mother plants. The first group included male and monoecious Laboko plants and the second group mainly Katala plants and female Laboko plants. Hormones were also divided into two main groups. The first included ABA and its derivatives, IAA metabolites, BzA, JA precursor and CKs metabolites which were generally more concentrated in female plants. The second group included bioactive JA, SA, bioactive auxins, bioactive CKs and precursors, which were generally more concentrated in monoecious plants of both cultivars and in male Laboko plants. Fig. 5 describes the results for each type of hormone in more detail.

Overall, the concentration of ABA-types was not affected by tuber region (F = 1.00, P = 0.3783) but varied with the sex of the mother plant (F = 11.75. $P < 0.0001$, Fig. 5A-B). Thus, regardless of tuber regions, concentrations of ABA-types were higher in tubers of female plants than in tubers of male and monoecious plants, regardless of cultivar (Fig. 5A-B). However, differences were observed between tuber regions when each sex was analysed separately. In Katala, the highest concentration of ABA-types was found in male plants in the middle region, in female plants in the head region, and in monoecious plants in the base region (Fig. 5A). In Laboko, no differences were found between tuber regions in male and monoecious plants, but the concentrations of ABA-types was higher in female plants in the middle region than in the head regions of the tubers.

In contrast, auxin concentration was not affected by tuber region (F = 1.14, P = 0.3160) or mother plant sex (F = 1.19, P = 0.3160, Fig. 5C-D) in either cultivar.

For SA, total concentration was not influenced by tuber regions (F = 2.29, P = 0.1187), but rather by mother plant sex (F = 4.00, P = 0.0008), although there was a significant effect of the sex-region interaction (F = 3.31, P = 0.0231; Fig. 5E - F). In Katala, the concentration of SA was higher in the tubers of monoecious plants than in the

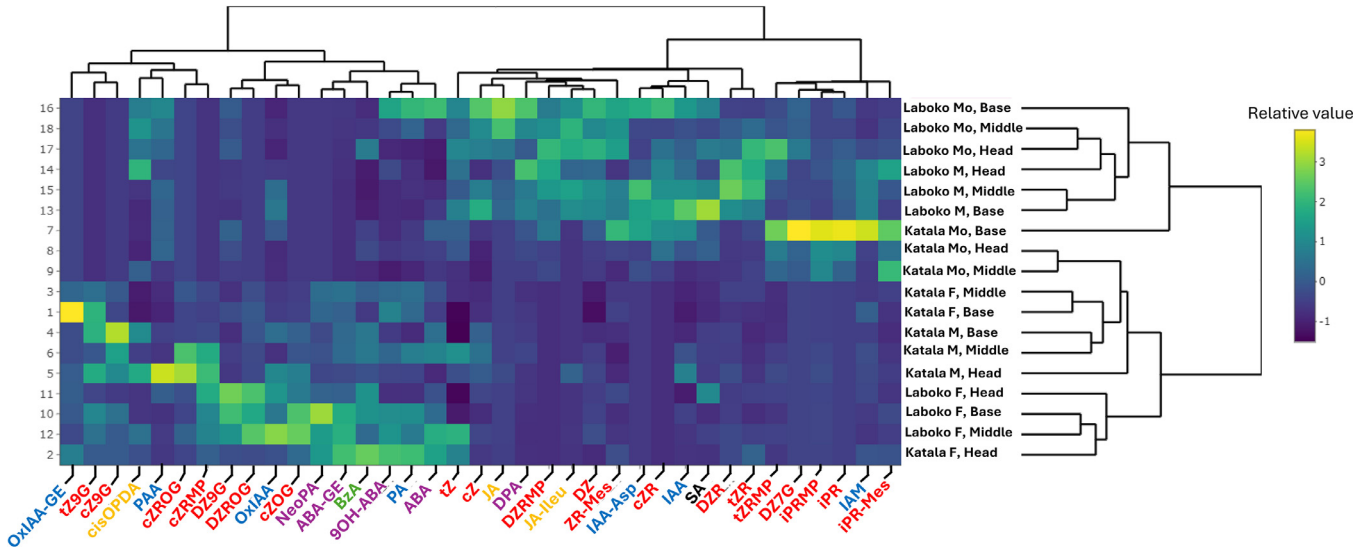

Fig. 4. Heatmap of the hormonal profile in yam tubers of *D. rotundata* cv. Katala and Laboko according to the sex of mother plant (M for male, F for female, and Mo for Monoecious) and the tuber region (head, middle, base). The compounds are colored according to the type of hormone: abscisic acid and related compounds in purple (ABA = abscisic acid, NeoPA = neophaseic acid, ABA-GE = ABA-glucose ester, 9OH-ABA = 9-hydroxy-ABA, PA = Phaseic acid, DPA = dihydrophaseic acid,); auxins in blue (IAA = indole-3-acetic acid, IAA-GE = IAA-glucose ester, PA Phenolic acid, OxIAA = oxo-IAA, IAA-Asp = IAA-aspartate, IAM = Indole-3-acetamide); salicylic acid in black (SA= Salicylic acid), benzoic acid in green (BzA = benzoic acid), jasmonates in yellow (JA = jasmonic acid, cisOPDA = cis-Oxophytodienoic Acid, JA-Ile = JA-isoleucine), cytokinins in red (tZ9G = trans-zeatin-9-glucoside, cZ9G = cis-zeatin-9-glucoside, cZROG = cis-zeatin riboside -O-glucoside, cZRMP = cis-zeatin riboside monophosphate, DZ9G = dihydrozeatin-9-glucoside, DZROG = dihydrozeatin riboside -O-glucoside, cZOG = cis-zeatin-O-glucoside, tZ = transzeatin, cZ = ciszeatin, DZRMP = dihydrozeatin riboside monophosphate, DZ = dihydrozeatin, ZR-MeS = 2-methylthio zeatin riboside, DZR = dihydrozeatin riboside, tZR = trans-zeatin riboside, tZRMP = trans-zeatin riboside monophosphate, DZ7G = dihydrozeatine-7-glucoside, iPRMP = isopentenyl adenosine monophosphate, iPR = isopentenyl adenosine, iPR-MeS = 2-methylthio isopentenyl adenosine).

tubers of both male and female plants (Fig. 5E), whereas in Laboko, the concentration of SA was higher in the male plants than in the female plants (Fig. 5F).

For BzA, another phenolic compound besides SA, concentrations were not dependent on tuber region ($F = 3.35$, $P = 0.0584$), but could vary with mother plant sex depending on cultivar ($F = 5.31$, $P = 0.0105$; Fig. 5G – H). Indeed, total BzA concentration was higher in female compared to male and monoecious plants in Laboko (Fig. 5H), whereas no difference in BzA concentration between sexes was observed in Katala (Fig. 5G). In addition, a significant effect of the interaction between sex and region was observed ($F = 3.13$, $P = 0.0288$), although no differences were found between tuber fragments when sex and cultivar were analysed separately (Fig. 5G – H).

The concentration of jasmonates (JA and its derivatives) was not affected by the tuber region ($F = 0.93$, $P = 0.4043$), but was strongly influenced by the sex of the mother plant ($F = 16.18$, $P < 0.0001$). In Katala, tubers from male plants had a significantly higher concentration of jasmonates than tubers from female plants, and monoecious plants had an intermediate of concentration jasmonates (Fig. 5I). In Laboko, the concentration of jasmonates was higher in tubers from monoecious plants than in tubers from both male and female plants (Fig. 5J). Comparison between cultivars showed that the concentration of jasmonates was 3-fold, 2.5-fold and 37-fold higher in Laboko tubers than in Katala tubers in male, female and monoecious plants, respectively ($P = 0.0010$; 0.0112 and < 0.0001, respectively). A significant effect of the interaction between sex and region ($F = 5.31$, $P = 0.0105$) was also observed. In Katala, the concentration of jasmonates in the head was higher than in the other tuber regions in female plants, whereas no differences were observed between tuber regions in male and monoecious plants (Fig. 5I). In Laboko, no differences were observed between tuber regions regardless of the plant sex (Fig. 5J).

Finally, the total concentration of CKs, like that of the other hormones, did not depend on the region of the tuber ($F = 1.14$, $P = 0.3160$, Fig. 5K - L). On the other hand, sex had a significant effect on CKs tuber concentration ($F = 39.96$, $P < 0.0001$), and the interaction between sex and region was significant ($F = 7.48$, $P = 0.0023$). Indeed, CKs concentration was higher in tubers of female plants than in those of monoecious plants in both cultivars, although CKs concentration was similar in tubers of male and female plants in Katala and in tubers of male and monoecious plants in Laboko (Fig. 5K and L). Moreover, CKs concentration was higher in the head region of female plants and in the base region of monoecious plants in Katala than in the other tuber regions (Fig. 5K), while it was higher in the middle region than in the head region of female plants in Laboko (Fig. 5I). However, tuber region had no effect on CKs concentration in male plants, regardless of cultivar (Fig. 5K - L).

4. Discussion

4.1. *The tuber region affected the germination and the flowering of* D. rotundata

This study showed that the region of the tuber used as seed-tuber influenced the germination rate and germination time in *D. rotundata*. Overall, seed-tubers from the head region germinated earlier with a higher germination rate than those from the middle and base regions. The same observation was made by Zoundjihekpon et al. (1995) in this species. These authors observed the existence of a germination gradient from the head to the base of the tuber, although the difference between the middle and base sections was not always significant for all cultivars. Kossou (1990) also found a difference in the viability of micro-fragments depending on whether they came from the apex or the basal part of the tuber. The viability of these micro-fragments was also influenced by environmental factors such as the chemical composition of the soil and the presence of organic matter (Kossou, 1990). A similar germination gradient along the tuber was also observed in the related yam species *D. batatas, D. trifida, D. cayenensis* and *D. alata* so that fragments from the apex of the tuber always germinated earlier than those from the middle and base (Sawada, 1952; Miège, 1957; Mathurin and Degras, 1975). In *D. cayenensis* and *D. alata*, Miège (1957) explained this germination gradient by an uneven distribution of starch grains and sometimes raphids along the tuber. Mathurin and Degras (1975) confirmed the longitudinal heterogeneity of the tubers in *D. trifida* and *D. alata* and its

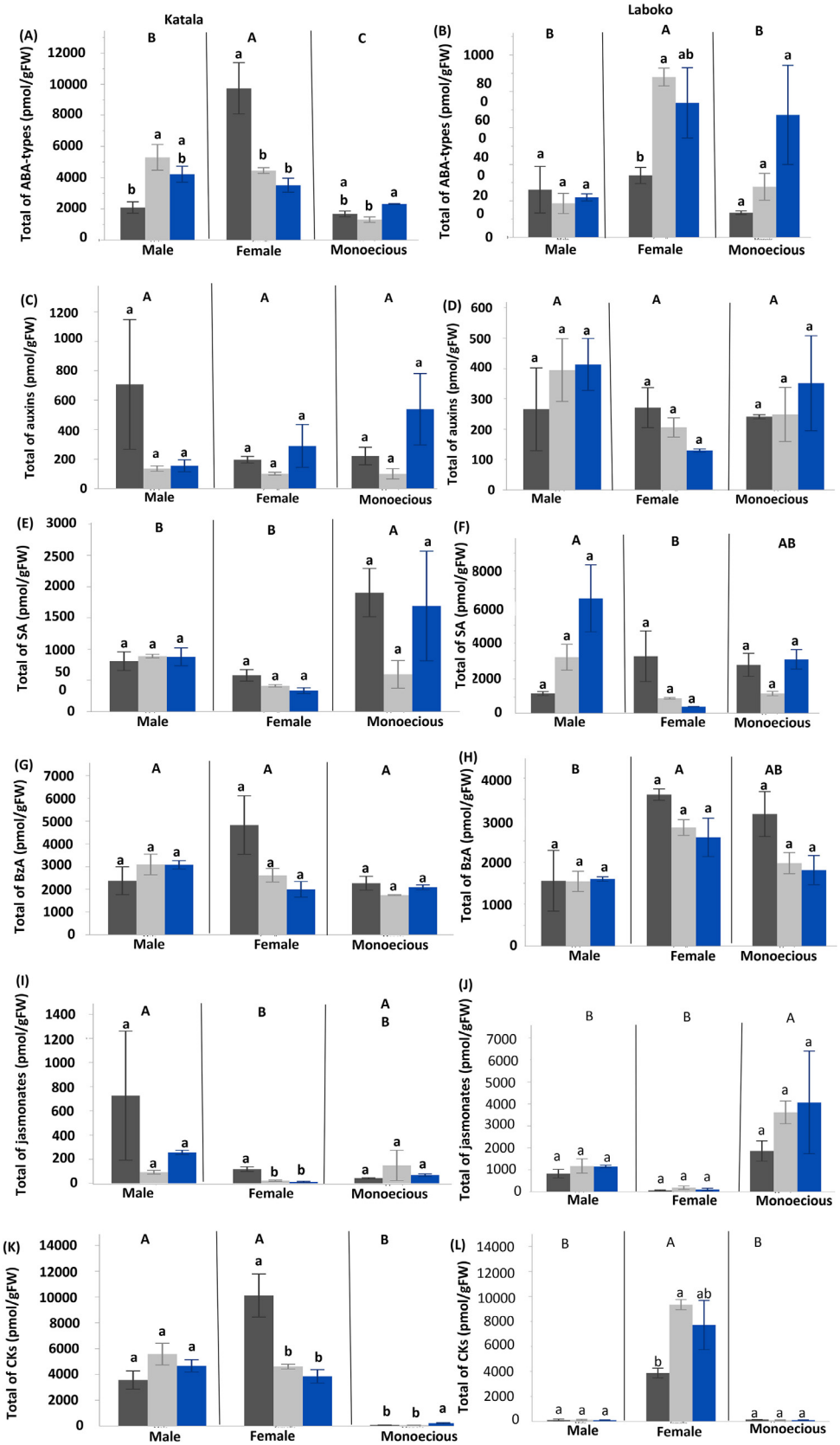

Fig. 5. Influence of tuber region (head, middle, base) and sex of mother plant on hormonal profile of yam tubers of *D. rotundata* cv. Katala and Laboko. Concentrations of (A - B) abscisic acid-types (Total of ABA-types), (C - D) auxins (Total of auxins), (E - F) salicylic acid (Total of SA), (G - H) benzoic acid (Total of BzA), (I - J) jasmonates (Total of jasmonates), (K - L) cytokinins (Total of CKs). For the same sex, regions having the same lowercase letter are significantly similar at the 5 % level. For the same cultivar, sexes having the same uppercase letter are significantly similar at the 5 % level. Data are means ± SE.

impact on the germination of the fragments (tuber-seeds). Apart from these factors, the high germination rate and the early emergence of the nodal region could be explained by the fact that, in general, the tuber head initiates buds mostly before sowing. These buds, which sometimes develop even before sowing (under certain temperature and humidity conditions), allow the tuber to germinate a few days after sowing with little risk of rotting, even if the tuber has been cut. Sawada (1952) made a similar observation in *D. batatas* and found that the size of the tuber could also influence the development of these buds. It should be noted that the middle tuber sections have to cope with the stress of the double scar (Fig. 1), leading to a high rotting rate, unlike the other sections which have only one scar. Pre-germination could help to cope with the stress of the double scar and increase the germination rate of seed-tubers from the middle region, as observed for the germination of minisets (Morse and Mcnamara, 2017).

Regarding flowering, our results showed an average flowering rate of 33 %, with flowering rates ranging from 50 % to 100 % depending on the cultivar, with the exception of Anago, which did not flower. This average value is lower than the average flowering rate of 70 % observed in other studies for *D. rotundata* from Benin (Tostain et al., 2005; Yolou et al., 2015). However, it is in the range of 25 % to 56 % of flowering observed by Zoundjihekpon et al. (1995) for this species in Côte d'Ivoire. Our results showed that the flowering rate was affected by the tuber region but that it mainly varied between cultivars. Indeed, depending on the cultivar and the sex, flowering can be weak or even absent. This was the case with Anago (female cultivar), which did not flower in the first trial of this study. This female cultivar, like most female plants, has been described as very demanding in terms of environmental factors (light intensity, soil quality, staking, etc.) for flowering induction and can produce very few flowers depending on the region of Benin where it is grown (Dansi et al., 1999; Yolou et al., 2015). We also did not observe flowering in this cultivar during our 2017–2018 field trial at the national collection field (N. Denadi, personal observation). Overall, Zoundjihekpon et al. (1995) reported that the flowering time of females in *D. rotundata* was about 2 times shorter than that of males. Our results showed that flowering time also varied between cultivars but not between tuber regions. Although flowering time was not affected by the plant sex in our study, it is known that there is a delay between male and female flowering in the cultivated yams *D. rotundata, D. alata*, and *D. dumetorun*, (Nwoke, 1991;Némorin, 2012; Yolou et al., 2015). However, the observation made in our study can be justified by the fact that the cultivar Alakissa is one of the few late flowering male cultivars. We also observed that the flowering time was negatively correlated with the germination time. Zoundjihekpon et al. (1995) also observed that in cultivated yam *D. rotundata* from Côte d'Ivoire, the seed-tubers that germinated last tended to be the first to flower. According to Lafon et al. (1988), in late-germinating plants, the vegetative buds were still growing when the environmental conditions allowed flowering and therefore did not compete with the flowering buds. In early germinating plants, however, the plants flowered when their vegetative growth was almost complete and the vegetative organs were extracting maximum sap to the detriment of the potentially flowering apices (Lafon et al., 1988). It should also be mentioned that the factors affecting flowering may differ between *Dioscorea* species and even between cultivars within the same species as reported by Hamadina et al. (2009). Depending on the flowering season or period, plants that germinated later will have a shorter growth period towards that season and will therefore be classified as having a shorter flowering period.

4.2. Sex variation in the offspring depended on the sex of the parent plant

Our results showed a variation in the sex of the offspring. This variation was not related to the tuber region, nor to the time of germination or flowering. It was rather related to the sex of the mother plants: the offspring of male and female plants showed little or no sex variation, whereas the offspring of monoecious plants showed high sex variation. We also observed that when sex variation did occur in the offspring, monoecious plants produced predominantly male offspring, whereas male and female plants produced predominantly monoecious offspring. Sex variation had previously been reported in *D. rotundata,* although the reason for this was not clear. Dansi et al. (1999) discussed the problem of sporadic sex variation in cultivated yams *D. rotundata*. They observed that two fragments of the same tuber from a female cultivar (Ahimon) produced male and female plants in the offspring (Dansi et al., 1999). We also observed sex variation in the offspring of the monoecious cultivars Katata and Laboko using minisets - from the same tuber - as seeds-tubers, meaning that all plants of the offspring were clones and shared the same genetic background (data not shown). Thus, our results suggest that the sex identity in *D. rotundata* is not only genetically determined, at least in the monoecious cultivars.

Tamiru et al. (2017) identified a genomic region associated with female heterogametic (male = ZZ, female = ZW) sex determination in *D. rotundata.* Plants with the ZZ genotype are male, while plants with the ZW genotype can be female, monoecious or non-flowering; W being dominant over Z would exert a so-called Z-suppressor effect on the latter (Tamiru et al., 2017). According to these authors, in females, if the Z-suppressor effect is complete, the offspring will be female, but if it is partial or null, the offspring will be monoecious or even male or non-flowering (Tamiru et al., 2017). According to this hypothesis (Tamiru et al., 2017), the sex variation we observed in the offspring of the monoecious plants would be explained by the strength of the Z-suppressor effect. Moreover, it has been shown that plants with the ZW genotype always flowered as males when their ploidy level was higher than 2X (Denadi et al., 2020; Girma et al., 2019). This could explain the lack of sex variation in the offspring of the male cultivars used in this study since Flou has a ZZ genotype and Alakissa is tetraploid (Denadi et al., 2020). However, it should be mentioned that other *Dioscorea* species such as *D. alata, D. floribunda* or *D. tokoro* showed a male heterogametic (male = XY, female = XX) sex determination (Cormier et al., 2019; Sugihara et al., 2021) and that our previous results suggest that the Beninese *D. rotundata* cultivars have rather adopted a male heterogametic system that is undergoing reorganisation (Denadi et al., 2020). Further studies are therefore needed to unambiguously identify sexual chromosome determination in *D. rotundata*. According to Perrin et al. (2009), and Rodrigues et al. (2017), occasional recombination between X and Y chromosomes could occur, allowing for periodic sex reversal. For example, homologies have been shown between the X chromosomes of monoecious hemp (*Cannabis sativa*) and the X and Y chromosomes of dioecious hemp (Faux et al., 2016). Hemp is a diploid species that includes both dioecious and monoecious cultivars and shows a high plasticity of sex expression (Faux et al., 2016), as we observed in *D. rotundata.*

Whether the sex identity is homo or heterogametic in *D. rotundata*, other internal and external factors must modulate sex expression. Golenberg and West (2013) proposed a model that can explain the evolution of dioecy through monoecy, and the mechanisms of environmental sex determination. According to them, key sex-determining genes must exist in monoecious species, but independent cues, either external environmental or internal physiological, must regulate their expression (Golenberg and West, 2013). Sex variation (masculinisation or feminisation) due to environmental factors (water stress, light intensity, shading, abundance of nutritive resources) has been reported in several monoecious and sometimes even dioecious species, including those with well differentiated sex chromosomes such as *Elaeis guineensis, Zea mays, Rumex hastatulus, R. nivalis, Cannabis sativa*, (Minina, 1938; Westergaard, 1958; Freeman et al., 1980; Rood et al., 1980; Adam et al., 2011; Orozco-Arroyo et al., 2012;). In *D. rotundata*, Tamiru et al. (2017) proposed that the Z-suppressing function could be affected by the environment, explaining

that plants with ZW genotypes could be female, monoecious, non-flowering or even male. Agre et al. (2020) confirmed that male or monoecious phenotypes could be observed in ZW genotypes instead of the female phenotype, depending on the growth environment. Mondo et al. (2023) have shown that agricultural practices such as pruning and tuber removal have masculinising and feminising effects on monoecious *D. rotundata* plants, respectively. Numerous studies have also documented the effects of hormones on alternative or plastic sexual development (Leite Montalvão et al., 2022; Niu et al., 2022).

As with environmental effects, many phytohormones have similar masculinising or feminising effects in independent species (Golenberg and West, 2013). The balance between gibberellins (GAs) and IAA plays an important role in sex determination in monoecious and dioecious species (de Jong and Bruinsma, 1974). Lee et al. (2019) reported that IAA and CKs play a key role in gynoecium formation, whereas GAs and JA or their combination are actively involved in stamen development and function (Marciniak and Przedniczek, 2019). It is also known that in some species hormonal treatment can influence the sex of flowers. This is the case in cucumber (*Cucumis sativus*), hemp (*Cannabis sativa*), spinach (*Spinachia oleracea*), or bitter melon (*Momordica charantia*), where IAA is feminising and GAs are more masculinising (Iwahori et al., 1970; Galoch, 1978; Akter and Rahman, 2010; Zhang et al., 2017; West and Golenberg, 2018). In a recent study, Mondo et al. (2023) showed that silver thiosulphate (STS) promoted the production of female flowers in *D. rotundata* monoecious plants while GAs did not have a strong effect on flower sex. Methyljasmonate has been shown to have a masculinising effect in *D. rotundata* monoecious plants (Denadi et al., 2024). In this study, we observed changes in the phytohormone profile of tubers in relation to the sex of the parent plant. However, we could not determine the sex of the offspring because the seed-tubers did not germinate (probably due to tuber dormancy or scar stress). According to our results, jasmonates, SA, BzA, ABA-types, and CKs could be potential candidates for sex modulation in cultivated yam *D. rotundata*. In the same cultivar, jasmonates and SA were more concentrated in monoecious and male plants, whereas CKs, ABA-typesand BzA were more concentrated in female plants. However, these results should be interpreted with caution as hormone concentrations may be subject to strong inter-individual variability. It would be interesting to see if a correlation could be made between the concentration of these hormones in the tuber and the sex of the plants produced, and if these hormones could influence sex expression in *D. rotundata*. It is also possible that the hormone levels in the tuber may vary depending on the earliness of the tuber (early or late harvest), and therefore a possible sex variation depending on the type of tuber (early or late) used as seed-tuber. Further research is therefore needed to investigate the hormonal control of sex variation in *Dioscorea rotundata.*

5. Conclusion

Our study has shown that, in *Dioscorea rotundata* yams from Benin, spontaneous sex variation occurs in the offspring, especially in monoecious cultivars. To understand the cause of this instability, we investigated the impact of the tuber region used as seed-tuber, the cultivar, and the sex of the mother plant. Our results showed that plants originating from the tuber head germinated faster and that germination time affected flowering rate. Then, for better outcomes, we advise yam breeders to use seed-tubers from the head when they need high and early germination and high flowering rate. However, neither the region of the tuber nor the germination and flowering time were responsible for the sex variation in the offspring. Only the cultivar and the sex of the mother plant influenced the sex of the offspring. Examination of the phytohormone profile of the tubers revealed differences between the sexes within the same cultivar. Tubers from male and monoecious plants had higher total concentrations of jasmonates and SA, whereas tubers from female plants had higher concentrations of CKs, ABA-types and BzA. Our results suggest a relationship between the hormone profile of tubers and the sex of daughter plants, although this needs further investigation. In the future, it would be interesting to test whether these hormones could influence sex expression in *D. rotundata*. Indeed, control of sex determination is crucial for breeding programmes and for improvement of the species.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Narcisse Denadi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gabin Agban:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Aline Vanhove:** Writing – review & editing, Investigation, Formal analysis. **Elie Assaba:** Writing – review & editing, Investigation. **Petre I. Dobrev:** Writing – review & editing, Methodology, Investigation. **Václav Motyka:** Writing – review & editing, Methodology, Investigation. **Jeanne Zoundjihékpon:** Writing – review & editing, Supervision, Project administration. **Christophe Gandonou:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Muriel Quinet:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2025.04.033.

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