Incomplete penetrance in maize genotypes segregating for the polyembryony trait

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Abstract

To study the genetic control of polyembryony trait in maize germplasm a series of experiments were carried out. The genetic material came from crossings among two polyembryonic populations and 16 different genotypes, normal type maize. A total of 27 F_1 were generated in 2016, and from those, there were derived 22 F_2, and 20 backcrosses genotypes. The experiments were carried out in two locations in Northern Mexico. Several genotypes in the second generation progenies share the same preceding F_1. The theoretical expectations for polyembryony (PEm, in short) proportions in F_2 and backcrossing are 0.0625 and 0.25, respectively. It is instructive to state that given the PEm recessiveness, all the F_1 genotypes were normal type plants: one seedling per germinated seed. The statistical methods applied to the experimental data were the exact Binomial test, for the segregating proportions in F_2, and the exact Fisher test to prove for independence between environments and the PEm genotypes. There were used R procedures for calculations. Based on the results, we have concluded that 1) varying genetic backgrounds in crossings might have an impact on the trait segregation proportions depending upon the specific parents' genotypes, which eventually lead to a penetrance reduction of the PEm genes expression, 2) polyembryony frequencies of the two populations were always statistically the same, no matter the environmental conditions where they were grown, and 3) the trait´s inheritance model was validated.

Introduction

Polyembryony in maize (*Zea mays* L.) has been reported for more than a century only as a phenomenon but not for its agronomic potential. The first publications described this rare and elusive trait as "false polyembryony" given that caryopses could germinate in two or three plumules and even two primary roots, but solely one cotyledon (Schrenk, 1894; Kempton, 1913; Weatherwax, 1921; Kiesselbach, 1926; Randolph, 1936).

Maize polyembryony (PEm, herein and after) has been reported mostly as a spontaneous mutant. However, there are some papers dealing with induced polyembryony by mutations (Morgan and Rappleye, 1951; Erldeská and Vidovenková, 1992). The former authors worked applying X-rays on pollen grains and evaluated its effect on the progenies; meanwhile the second paper reports the effect of the chemical agent 2-4-Dichlorophenoxiacetic acid (2-4-D) in caryopsis two days after pollination, to induce successfully the cleavage polyembryony type.

During the last quarter of the 20th century, a few reports on PEm were published. One is from Pesev *et al*. (1976) who derived a set of inbred lines from a synthetic maize variety that showed ears with kernels with two and three embryos. The polyembryony frequency in the derived inbred lines ranged from 2.1 to 25.3 % which might be a clue about the incomplete penetrance for the PEm trait, despite of the homozygosis generated by inbreeding.

Two other papers on polyembryony are from Castro and Rodriguez (1979) and Espinoza *et al*. (1998). The former reported a heritability estimate of 69 % for the "twin seedlings" trait, observed in a maize population named as "super dwarf selection" (SSE, in short). The last authors reported an average frequency of 60 % on the trait after 14 recurrent selection cycles in two evolving populations derived from the SSE population.

The term "polyembryony" referring to maize can be found in some papers, like the ones reported by Kermicle (1969), Erdelská and Vidovencová (1992), Espinoza *et al*. (1998), Rebolloza *et al*. (2011), Espinoza *et al*. (2012), Alcalá *et al*. (2019). It is very instructive the paper by Erdelská (1996) from which it is quoted that "the histological analysis enables to distinguish the different types of polyembryony in maize caryopses connected with the different origin of twins or triplets." The author stated that "the crucial differences" of PEm types can be due to the localization, and structure of embryos, as well as the type of germination of the caryopses.

Nowadays, the maize polyembryony is considered as an interesting but elusive phenomenon (Michel, *et al.*, 2018). Given that the tag as a quantitative trait no longer holds, there are two proposals about the trait's nature. The first, reported by Rebolloza *et al*. (2011) stated that the inheritance of PEm is due to two interacting loci, duplicate dominant epistasis (15:1 ratio) type. The second, reported by Meraz-Fonseca *et al*. (2015) proposed that the "tallos gemelos" trait shows evidences of epigenetic mechanisms for its genetic control.

Whatever the nature of PEm might be, the fact is that the trait is genetically controlled, and so far, no one had reported complete penetrance for the trait. To the contrary, all known reports on the trait's frequency have declared mean values lower than 70 %, which might be an indication of an incomplete penetrance of the gene or genes that control the trait, provided that penetrance in genetics is the proportion of individuals carrying a particular variant of a gene or genes that also express an associated phenotype (Pesev *et al.*, 1976; Castro and Rodríguez, 1979; Espinoza *et al.*, 1998; Rebolloza *et al.*, 2011; González *et al.*, 2011; Espinoza *et al.*, 2012; Meraz-Fonseca *et al.*, 2015; Alcalá *et al.*, 2019).

In Genetics, it is well known that the term "penetrance" quantify the modification of gene expression by varying the environment, and/or the genetic background. They measure respectively the percentage of cases in which the gene is expressed and the level of expression (Griffiths *et al.*, 2015).

There are reports on partial or incomplete penetrance of major genes of diverse effects in maize. About gene(s) for grain color, Sekhon and Choppa (2009) informed that the gene "unstable factor for orange1" (*Ufo1*), whose induced phenotypes are not completely penetrant such that only a subset (27%) of F_1 progeny (P1-wr, pericarp color 1-white pericarp, red cob glumes; *Ufo1*) plants shows gain of pericarp pigmentation. A gene related to maize reproduction, Barret *et al.* (2008) reported the gene ggi1, which is an inductor of gynogenesis in maize, has a penetrance lower than 41 % in segregating F_2 progeny.

The present research work is aimed to get experimental data that might allow the authors to grasp some explanation on the incomplete penetrance phenomenon, probably associated to the polyembryony in maize. Additionally, and given that one of the proposed model for maize polyembryony inheritance is through the action of two loci with dominant duplicate type of epistasis, this work is also intended to validate the 15:1 segregating proportion in the F_2's

Objectives

To generate all possible crosses (F_1's) among two maize polyembryonic populations and 16 different genotypes of normal maize, and the subsequent F_2's progenies obtained as maternal half sibs. The plan also includes a series of back crossings (BC's) from the F_1's genotypes to both BAP and NAP populations.

To test for the polyembryony proportions in each of the three kinds of progeny, taking into account the expected proportions as follow: 0 polyembryony in all the F_1's; 0.0625 in the F_2 groups; and 0.25 in the BC groups.

To get some measures of the amount of the incomplete penetrance associated to the polyembryony phenomena.

To test for environment – genotype independence because of different locations where the polyembryonic genotypes were grown.

Materials and Methods

Genetic material

The maize genetic material used in this work is shown in Table 1. The two polyembryonic populations, the AN's inbred lines and the two AN's commercial hybrids (CoHy AN-), were developed at the Instituto Mexicano del Maíz "Dr. Mario Castro Gil"- Universidad Autónoma Agraria Antonio Narro (IMM-UAAAN, in short). On the other hand, the CML's and the Tuxpeño HOC population were provided by CIMMYT, and the commercial hybrids (CoHy) are sold for diverse seed companies operating in México.

Field experiments

The experimental work started in the summer of 2016 at the "Buenavista" (BV) location; with geographical coordinates 25° 22' N, 101° 02' W, 1742 meters above the sea level (masl), annual mean temperature 16.8 °C, and 350-400 mm accumulated rainfall. To begin with,

Table 1: Maize genetic material

ID	Description	Source
BAP = D	IMM-UAAAN-BAP, Brachytic maize population, with a PEm average frequency of 60 – 65 %	Instituto Mexicano del Maíz, "Dr. Mario Castro Gil", Universidad Autónoma Agraria Antonio Narro, headquarters in Saltillo, México. (IMM-UAAAN).
NAP = C	IMM-UAAAN-NAP, Normal height maize population, with a similar PEm frequency as in BAP.	The same as in BAP.
Tuxpeño-HOC population	Maize population high in grain oil content, about 8 %. Normal type maize.	Sample provided by CIMMYT (‡ exotic)
AN-255-18-19	Dwarf maize inbred line, No-PEm.	IMM-UAAAN (exotic)
AN-ML-S4-1	Dwarf maize inbred line, No-PEm.	IMM-UAAAN (exotic)
AN-TEP-3	Normal height inbred line, No-PEm.	IMM-UAAAN (exotic)
AN-CS-8	Normal height inbred line, No-PEm	IMM-UAAAN (exotic)
AN-RBV-1	Normal height inbred line, No-PEm	IMM-UAAAN (exotic)
AN-Tuxpita	Normal height inbred line, No-PEm	IMM-UAAAN (exotic)
AN-MJ-2	Normal height inbred line, No-PEm	IMM-UAAAN (exotic)
CML-332	Normal height inbred line, No-PEm	Sample provided by CIMMYT ("exotic")
CML-264	Normal height inbred line, No-PEm	Sample provided by CIMMYT (exotic)
CoHy Garañón	Commercial hybrid, normal maize type, No-PEm	Asgrow–Monsanto-Bayer, widely commercialized in Mexico (exotic)
CoHy DK-4060	Commercial hybrid, normal maize type, No-PEm	Dekalb-Monsanto-Bayer, widely commercialized in Mexico (exotic)
CoHy 30G49	Commercial hybrid, normal maize type, No-PEm	Pionner-Dupont, widely commercialized in Mexico (exotic)
CoHy H-437	Commercial hybrid, normal maize type, No-PEm	INIFAP, a Public Agricultural Livestock and Forestry Research Institute in Mexico (exotic).
CoHy AN-388	Commercial hybrid, normal maize type, No-PEm	IMM-UAAAN (exotic)
CoHy AN-447	Commercial hybrid, normal maize type, No-PEm	IMM-UAAAN (exotic)

‡ Exotic = Normal maize genotypes, with no relationship to the two PEm populations.

the 18 initial genotypes were developed along May to October, 2016. The plan was to generate crossings among the two PEm populations (BAP and NAP) with the 16 other genotypes (exotics), direct and reciprocals crossings when possible, in order to get several F_1's, with 50: 50 % germplasm combinations (PEm: Exotics). The plot size for the populations was 14 rows, 0.8 m apart, 24 m long, and 20 cm between plants. All other materials were sowed in plots of 3 rows, 0.8 m apart, 10 m long, and 15 cm between plants.

At harvest, a total of 59 different F_1 genotypes were obtained, but only 27 were selected following the criterion of progeny size, requiring at least 2000 seeds per crossing. Also, a new generation of BAP and NAP was obtained, contemporaries of those F_1's.

In order to generate the F_2 progenies, the selected F_1 were sowed at the "Rio Bravo" (RB) location (25° 58' N, 98° 06' W, 30 masl, annual mean temperature of 22.6 °, and 648 mm rainfall). Location RV is 340 Km apart from BV, following the federal high way 40D to the Northeast in Mexico. The experiment was developed during the February – June, 2017 cycle. Each F_1 genotype was planted in two replicates, plots of 3 rows, 0.8 m apart, 5 m long. At flowering, a mixture of pollen of each F_1 genotype collected in one replicate was used to pollinate silks in the other replicates and vice versa.

Table 2: F_2 progenies, seedling stage, from 22 genotypes segregating the polyembryony trait under the hypothesis 15:1.

ID	Initial crossing = F_1	Total F_1 seedlings	No. of PEm seedlings	*f* (PEm) %	*p*-value
a	C[‡] x AN-255-18-19	285	24	8.4	0.1402
b	C x AN-ML-S4-1	291	8	2.7	0.0104
c	C x AN-CS-8	278	24	8.6	0.1062
p	C X AN-Tep-3	292	9	3.1	0.0212
d	C x AN-RBV-1	291	17	5.8	0.9035
e	C x AN-Tuxpita	275	11	4.0	0.1349
f	D[‡] x AN-255-18-19	289	12	4.2	0.1793
h	D x AN-CS-8	276	18	6.5	0.8035
i	D x AN-Tep-3	295	10	3.4	0.0403
j	D x AN-RBV-1	288	16	5.6	0.7154
k	D x AN-Tuxpita	288	13	4.5	0.2721
l	C x CoHy[‡] Garanón	290	15	5.2	0.5435
m	C x CoHy H-437	265	14	5.3	0.6117
o	C x CoHy AN-447	281	14	5.0	0.4591
q	C x CoHy DK4060	282	9	3.2	0.0352
r	D x ComHy Garanón	295	14	4.7	0.3358
s	D x ComHy AN-447	290	14	4.8	0.3945
t	ComHy DK-4060 x D	285	12	4.2	0.1778
u	ComHy H-437 x D	284	12	4.2	0.1776
v	ComHy 30P49 x D	289	23	8.0	0.2237
x	C x CML-334	287	13	4.5	0.2718
y	D x CML-334	285	15	5.3	0.6233
	General mean	285.5	14.4	5.04	
	Summed data, all genotypes	6281	317	5.05	$5.301e^{-05}$

[‡] C and D = short names of NAP and BAP populations respectively.[‡] CoHy and CoHyAN = commercial hybrids.

Also, at the RB location, samples of the BAP and NAP populations were sowed in plots with 27 rows, 0.8 m apart, 5 m long, and 20 cm between plants. The development of BAP and NAP were used as checks for PEm frequencies.

The pollination made on the 27 F_1 at the RB location led to only 22 selected F_2 genotypes, because of the same criterion of getting a minimum of 2000 seeds for each F_2 progeny. Also, it was recorded the PEm frequencies for BAP and NAP populations.

Back into the BV location, two experiments were established, as follows. 1) The F_2´s seed samples from the 22 selected genotypes were sown in plots of 6 rows, 0.8 m apart, 7 m long, with 11 cm between plants to have a measure of the segregating polyembryony; and 2) Samples from the two polyembryonic populations and 42 of the former 2016 F_1's were sown in order to generate backcrossing progenies to the PEm populations. Both experiments were established to the open field, close to each other, and developed along the period from early July to mid-December, 2017.

All the experimental genotypes, studied across locations and years were handled under irrigation, using a simple tape drip irrigation system. The general fertilization formula was 160:80:00 for N: P: K units, respectively, and the chemical fertilizers were Mono-

Table 3: F_2 progenies, flowering stage, belong to 22 genotypes segregating the polyembryony mutant under the hypothesis 15:1.

ID	$F_2 = (F_1)^2$	Total F_2 plants	No. of PEm plants	∫ (PEm)	*p*-value
a^2	$[C^{\dagger}$ x AN-255-18-19$]^2$	271	16	5.9	1
b^2	[C x AN-ML-S4-1$]^2$	264	6	2.3	0.0047
c^2	[C x AN-CS-8$]^2$	237	12	5.1	0.5899
p^2	[C X AN-Tep-3$]^2$	257	9	3.5	0.0706
d^2	[C x AN-RBV-2$]^2$	283	17	6.0	1
e^2	[C x AN-Tuxpita$]^2$	280	14	5.0	0.4588
f^2	$[D^{\dagger}$ x AN-255-18-19$]^2$	304	21	6.9	0.6345
h^2	[D x AN-CS-8$]^2$	276	16	5.8	0.9008
i^2	[D x AN-Tep-3$]^2$	277	9	3.2	0.0346
j^2	[D x AN-RBV-2$]^2$	292	10	3.4	0.0515
k^2	[D x AN-Tuxpita$]^2$	323	13	4.0	0.1071
l^2	[C x CoHył Garanón$]^2$	322	9	2.8	0.0076
m^2	[C x CoHy H-437$]^2$	257	12	4.7	0.3659
o^2	[C x CoHy AN-447$]^2$	300	17	5.7	0.811
q^2	[C x CoHy DK-4060$]^2$	325	15	4.6	0.2523
r^2	[D x CoHy Garanón$]^2$	309	14	4.5	0.2402
s^2	[D x CoHy AN-447$]^2$	301	15	5.0	0.4734
t^2	[CoHy DK-4060 x D$]^2$	314	6	1.9	0.0004
u^2	[CoHy H-437 x D$]^2$	299	9	3.0	0.0164
v^2	[CoHy 30P49 x D$]^2$	287	14	4.9	0.3936
x^2	[C x CML-334$]^2$	277	19	6.9	0.6201
y^2	[D x CML-334$]^2$	293	6	2.0	0.0014
	General mean	288.5	12.7	4.4	
	Summed data, all genotypes	6348	279	0.0439	$1.66e^{-10}$

ammonium phosphate, MAP (11: 52: 00, %) and Urea (46: 00:00, %), which were applying 80:80:00 at sowing, and 80:00:00 at the V8 stage.

Greenhouse experiments

Additionally, to the field experiments, a series of trials under greenhouse conditions were carried out to measure seedling characteristics, focusing on the PEm segregation frequencies. The studied genotypic groups were as follows, all the 59 F_1's, the selected 22 F_2's, and 20 backcrosses. The sowing was done in polyurethane "seed tray 200 square", with dimensions of 68 x 34 x 6 cm. The substrate was a mixture of forest soil and peat moss, 2:1 v/v. The daily temperature average was 28° ± 2 Celsius, and the temperature oscillation average was 12 degrees. The watering was manually on daily bases for a period of 15-17 days, at the time when the seedlings evaluation took place. In all the three experiments, the sample size was of 300 seeds by genotype.

Statistical analysis

The statistical method applied for testing the hypothesis in F_2's and BC's about the segregating proportions of PEm was the exact test of goodness-of-fit, also named the "exact binomial test", which is the proper method when there is one nominal variable, and sample sizes lower than a 1000. Moreover, having two locations

Table 4: PEm frequency and the exact binomial test for backcrosses among the F_1 and the two PEm populations, at seedling stage.

New ID‡ = BAP or NAP x F_1's ID '	Backcross detail	Total BC seedlings	No. of PEm seedlings	∫ (PEm)	*p*-value
03 = BAP x d	D x (C x AN –RBV-2)	297	76	25.6	0.8407
07 = BAP x y	D x (D x CML-334)	297	75	25.3	0.9466
09 = BAP x v	D x (CoHy 30P49 x D)	293	78	26.6	0.5437
10 = BAP x i	D x (D x AN -Tep-3)	295	36	12.2	8.05e-08
21 = BAP x c	D x (C x AN- CS-8)	274	62	22.6	0.4025
23 = BAP x t	D x (CoHy DK4060 x D)	289	70	24.2	0.6873
24 = BAP x x	D x (C x CML-334)	292	50	17.1	0.0015
26 = BAP x l	D x (C x CoHy Garañón)	292	51	17.5	0.0023
28 = BAP x j	D x (D x AN-RBV-2)	277	64	23.1	0.4886
30 = BAP x p	D x (C x AN -Tep-3)	291	51	17.5	0.0028
13 = NAP x d	C x (C x AN-RBV-2)	292	73	25.0	1
17 = NAP x y	C x (D x CML-334)	296	71	24.0	0.7373
19 = NAP x v	C x (CoHy 30P49 x D)	299	76	25.4	0.8937
20 = NAP x i	C x (D x AN -Tep-3)	257	40	15.6	0.0003
31 = NAP x c	C x (C x AN – CS-8)	296	86	29.1	0.1076
33 = NAP x t	C x (CoHy DK-4060 x D	291	80	27.5	0.3431
34 = NAP x x	C x (C x CML-334)	228	54	23.7	0.7023
36 = NAP x l	C x (C x CoHy Garañón)	293	52	17.7	0.0036
38 = NAP x j	C x (D x AN-RBV-2)	293	73	24.9	1
40 = NAP x CH	C x (C x AN -Tep-3)	285	47	16.5	0.0008
	General mean	286.4	63.3	22.6	
	Global, Summed data	5727	1265	22.1	2.685e^{-07}

‡ The new ID for each BC is the two digits number. ‡ It must be remembered that D and C that appears in the second column are the short name of BAP and NAP respectively.

and years, the Fisher Exact test was applied to prove the independence between the environments and the PEm phenomenon. There were used R procedures for calculations (R Core Team, 2018).

Results and discussion

F_2's Polyembryony frequencies, seedlings and adult plants

The proportions of PEm, and the probability values after applying the exact binomial test for each F_2 progenies are shown in Table 2 (seedling stage), and Table 3 (adult plants at flowering). It is instructive to state that all the F_1 progenies germinated as normal maize type, i.e. each grain germinates in a single seedling with a simple plumule, validating the recessive nature of this PEm (Rebolloza *et al.*, 2011; Alcalá *et al.*, 2019), and with a general average of 96.3 % of seedlings emerged, at V2 or V3 stage (adopting the Ritchie *et al.* (1992) staging system to identify stages of corn development).

As shown in Table 2, most of the segregating genotypes fitted the expected PEm proportions, exceptions made with those in *b, p, i,* and q genotypes, whose PEm frequencies were low enough to be statistically rejected compared to the expected proportions, in spite of the positive non-cero value they shown. These genotypes' positive low values are of some importance if someone is looking for the possible mechanisms that affect the PEm expression. Also, it is relevant to notice that the inbred line AN-Tep-3 is the male parent of the p and i crossings, and no matter if NAP or BAP is the female parent, their progenies fail to show the

Table 5: Tracking to all genotypes that failed to meet the expected proportions appropriate to each of the three PEm segregating general groups (F_2 seedlings and adults, and BC's), identifying the common exotic germplasm source.

	PEm number / Total simple size			p-value		
New ID‡	F_2 Seedlings	F_2 Adult plants	BC's	F_2 Seedlings	F_2 Adult plants	BC's
10	10 / 295	9/277	36 / 295	0.0403	0.0346	8.047e-08
24	13 / 287	19/277	50/292	0.2718	0.62	0.0014
26	15 / 290	9 / 322	51 / 292	0.5435	0.0076	0.0023
30	9 / 292	9 / 257	51 / 291	0.0212	0.0706	0.0028
20	10 / 295	9/277	40 / 257	0.0403	0.0346	0.0003
36	15 / 290	9/322	52 / 293	0.5435	0.0076	0.0036
40	9 / 292	9 / 257	47 / 285	0.0212	0.0706	0.0007
PEm mean frequencies:						
	3.9 %	3.2 %	16.3 %			

‡ It is recommended to check the complete ID in Table 4.

expected Normal: PEm proportions. So, two points can be stressed from here, 1) the PEm is obstructed, but not totally inhibited, and 2) the pollen source might have an obstructing effect on the PEm expression in F_2 segregates.

On the other hand, the global or summed data across genotypes (bottom of Table 2) led to a calculated PEm frequency around 5 %, which was short to the expected 6.25 %. The very low calculated p-value can be taken as the evidence for the failure to reach the expected 15: 1 proportions, even though the fact those 18 out of 22 genotypes segregated accordingly to the expected value.

If the failed 4 out 22 genotypes are removed, and the global data set is analyzed with the 18 fitted genotypes, the sample size results in 5121 individuals, but only 281 are PEm, that's to say a mutant frequency of 5.49 %. The exact binomial test for such data will provide a *p*-value of 0.0243, which fall out of the acceptance area. This situation might be considered as an evidence for the incomplete penetrance of the PEm genes given that 9 out the 18 fitted genotypes showed PEm proportions in between 0.04 and 0.05, which represent solely 60 to 80 percent of the expected 0.0625. Actually, if one applies the test to the global data using only the nine genotypes with PEm frequencies above the general mean (Table 2), the total seedlings number is 2547, but only 166 are phenotypically polyembryonic. The calculated PEm frequency and *p*-values are 6.51 %, and 0.5665, respectively, which supports a 15:1 segregating PEm proportions.

These results might be an indication about the uncertainty of certain crosses among BAP or NAP with diverse exotic genotypes in regard to the PEm mutant segregation in F_2. The uncertainty might be referred to the concomitant incomplete penetrance phenomenon. The rejected 15:1 hypothesis in the b, p, i, and q genotypes are special situations about varying the genetic background. The interference in the PEm phenotypes' expression in the four failed genotypes might represent a partial penetrance in the range of 43 to 54 %, values that are in agreement to the ones published by Rebolloza *et al.* (2011), and Alcalá *et al.* (2019).

The PEm data from the tasseling-silking stage (Table 3) show that 7 F_2 out of 22 were rejected under the hypothesis about the PEm expected segregation of 1/16; this number is almost twice as the one in the seedlings experiment (Table 2). It is convenient to make some comments about differences between the two experiments, 1) although the number of seeds sowed in the field was larger than in the greenhouse (382 vs. 300 seeds), the germination percentage was lower (75 vs. 95 %), and 2) in the field experiment, the plants usually take more risks because of the use of mechanized labors as tillage, fertilizing, and weed control. The mechanical stroke might affect the number of plant per plot and/or the type of plants that are damaged, altering in some way the counts and proportions.

Differences about agricultural practices apart, the F2'S that showed a very low PEm frequencies in both plant stages were only the b and i, genotypes, however, it is worth to notice that the p genotype that was rejected in the F2 seedlings stage got a low enough probability level at the adult stage to be situated into the non-rejection side, this might be so because of sampling

Table 6: Data [‡] used for the Fisher´s exact tests, phenotypically classes PEm and Non-PEm, two locations, two years.

Populations/ Locations	Phenotypic Classes	Number of individuals	Populations/ Years	Phenotype Classes	Number of individuals
C BV 2017	PEm	676	CBV 2016	PEm	626
C BV 2017	Non-PEm	379	CBV 2016	Non-PEm	314
C RB 2017	PEm	339	CRB 2017	PEm	339
C RB 2017	Non-PEm	182	CRB 2017	Non-PEm	182
D BV 2017	PEm	718	DBV 2016	PEm	629
D BV 2017	Non-PEm	418	DBV 2016	Non-PEm	348
D RB 2017	PEm	330	DRB 2017	PEm	330
D RB 2017	Non-PEm	206	DRB 2017	Non-PEm	206

[‡] C and D = NAP and BAP populations. BV and RC = Locations Buenavista and Rio Bravo.PEm = Polyembryonic plants, Non-PEm = Single plumule per seed.

effect. The q genotype that failed at seedling stage, it was not rejected at the flowering stage, but the t genotype instead. It can be taken into account that both q and t genotypes have a parent in common, the CoHy DK4060 who eventually could affect the PEm proportion. Besides these four failed genotypes, there were find two other inbred lines (one from the AN group, and the other from the CIMMYT lines) that were crossed with BAP or NAP which progeny show a significant reduction in the penetrance capacity of the PEm genes. Also, it was registered that two other CoHy (Garañon and H-437) progeny when hybridizing with BAP or NAP showed a significant reduction in the PEm proportions. As it can be calculated, the seven rejected genotypes at adult stage showed an incomplete penetrance in the range of 32 to 54 %, quite similar to the ones rejected in the F_2 seedlings data (Table 2), and in accordance to the ones published by Rebolloza *et al.* (2011) and Alcalá *et al.* (2019).

When the summed data (table 3) is tested for the 15:1 hypothesis, once again it was found a very low p-value, which means a failure to meet the expected PEm frequency. If someone follows a similar data handling strategy as the ones explained for the F_2 seedlings data, it might find that from the 15 individually accepted genotypes, only six of them have a PEm frequency quite close to the expected 6.25 %, and the other nine, which are individually non-rejected but with low values, express the trait with partial penetrance, calculated in the range of 56 % to 82 %. Data similar to the one published by Rebolloza *et al.* (2011).

Table 7: Tests of independence among the population's polyembryony and environments (locations or years), and between the BAP and NAP.

Populations	Environments	Fisher test's *p*-value
BAP	Locations	0.5163
	Years	0.2893
NAP	Locations	0.7373
	Years	0.5644
BAP vs NAP	Between populations	0.4695

Backcrosses' Polyembryony Frequencies, Seedlings

The data relative to the backcrosses genetic analysis (BC's) is shown in Table 4. It is worth to notice that these data refer to cases where the F_1 genotypes were used as pollinators to both BAP y NAP, the recessive female genotypes.

The 20 backcrosses (BC's) are divided in two corresponding subgroups, 10 each in both female parents (BAP or NAP). Given the crossing plan, the resulting backcrosses carried a genome proportion of 75: 25 Polyembryonic: normal sources. In this context, the theoretical expectations for the Normal: PEm proportions were 12/16: 4/16, or simply 3: 1. Results showed that the range of the PEm frequency values across backcrosses were between 12.2 % and 29.1 %. Once again, most of the genotypes are in accordance with the expected proportions, but there was an important number of cases that failed. Looking in retrospective, it might be noted that 4 out of 22 in F_2 -seedlings, 7 out of 22, in F_2 -adult plants, and 7 out of 20 in the actual BC's were all rejected. Undoubtedly, in these types of crosses, some of them will be low enough to the expected PEm frequency and so statistically rejected, no matter if the segregates are

from an F_2 or a BC, and something very important in all these rejected genotypes, the PEm frequencies were always positive non-zero values.

When the summed data of the 20 BC's was subject to the statistical test, the ***p***-value was very low (Table 4). The seven cases of rejected segregating genotypes might have an impact to the global data lowering the PEm frequency. After the data from the seven rejected genotypes were removed, the resulting 12: 4 hypothesis test on the remaining non-rejected genotypes got a ***p***-value of 0.7725.

As it can be seen (Table 4), the seven BC's genotypes that didn't meet the theoretical expectations presented PEm frequency values in the range of 12.2 to 17.7 %, exhibiting the incomplete penetrance of the trait in calculated amounts of 49 to 71%. It must be aware that those genotypes who were statistically non-rejected presented a wide range of PEm frequency between 22 and 29 %, so that the complete penetrance is to be said that fell between 88 to 116 %, having the expected 25 % around the middle value of this range. The excess in PEm frequencies might be due to sampling errors, and/or to the complete compatibility among the genomes in crossings, so that there is a positive influence on the easiness to the polyembryony expression.

It is also clear that the BC's genotypes showed PEm proportions according to an inheritance model of two interacting epistatic loci of the "duplicate dominant" type, with a theoretical expectation of 12: 4 in BC1. Because of the BC's crossing nature, this proportion surpass about 4 times the segregation proportions 15:1 in the F_2 segregating genotypes. Results in the backcrossing procedure also validate the two loci model of the PEm inheritance proposed by Rebolloza *et al*. (2011).

The PEm segregation results from the BC's and F2'S groups can be compared about similarities and differences, for instance, the summed data in both two groups led to a rejection of the 12:4 and 15:1 segregating hypothesis, respectively, even though, most of the specific genotypes within group were non-rejected. On the other hand, it is remarkable that the penetrance percentages are higher in the first group, perhaps because of the BC's greater PEm germplasm doses (75: 25, instead the 50:50 in F2'S). With more PEm germplasm doses in the BC's genotypes, the reduced exotic germplasm that might interfere negatively to the trait gets less chance to affect the PEm segregation. As it is generally known, the reduced or incomplete penetrance can be a function of the specific mutation(s) involved or of allele dosage, or for differential allelic expression and that variable penetrance may also reflect the action of unlinked modifier genes, epigenetic changes or environmental factors. (Cooper *et al.*, 2013).

To check the consistency about the penetrance capacity on the PEm expression of some exotic genotypes across experiments (F2'S and BC's), the materials that repeatedly failed to fit the theoretical expectations, to be in proportions of 15: 1 or the 12:4 when crossing with BAP or NAP, were mostly the inbred line AN-Tep-3 and the CoHy Garañón (Table 5). However, it is not clear yet what kind of genetic mechanisms might have an impact to the reduction in penetrance on the two major loci that control the inheritance of the PEm trait. From the explanations that Cooper *et al.* (2013) have pointed out, we disregard the environmental factors because of the consistency of the PEm frequencies across locations, and lead to the possibility of the actions of unlinked modifiers genes, provided by some exotic materials.

It is worth to underline that BAP and NAP, which should be considered the putative populations of the PEm studied here, are genetic resources for the trait, described as highly polyembryonic materials, which means that they have not reached a 100 % frequency on the trait. The reported PEm average frequencies on both populations are between 55 % to 65 % (Espinoza *et al.*, 1998; Rebolloza et al,. 2011; Alcalá *et al.*, 2019). These phenotypically expressions of the PEm could reflect the partial penetrance of the trait, with a proportion average of 6/10.

The independence between PEm Frequency and varying environments

Given that the experiments were carried out across locations and years, the BAP and NAP populations were sowed every time because of their usefulness as crossing materials or just as polyembryony checks. To elucidate if the varied experimental conditions (environments) as one of the nominal variable had any effect on the second nominal variable (the polyembryony phenomena), a Fisher's exact test was applied. The data for analysis is presented in Table 6, and the test results are in Table 7.

The all-around PEm frequencies (across locations and years) were all in the range from 61.6 to 66.6 % which are in agreement with previous reports (Espinoza *et al.*, 1998; Rebolloza *et al.*, 2011; Gonzalez *et al.*, 2011; Alcalá *et al.*, 2019). Populations-wise, the calculated frequencies for D were from 61.6 to 64.4 %, and the ones for the C population were from 64.1 to 66.6 %, as stated before, all are pretty close to the expected 6/10 phenotypic expression of the mutant PEm.

The calculated probabilities for both locations and years resulted bigger enough to state that the phenotypically expression of the polyembryony trait was independent

from the environments where the PEm putative populations were grown. They also served to validate the equivalent amount about PEm frequencies in the two polyembryonic populations BAP or D, and NAP or C.

Conclusions

In a general context, the maize polyembryony phenomenon is a subject marginally studied. However, the findings in this research provided support to validate PEm mode of inheritance thought to be controlled by two epistatic loci, and also that PEm genes interact somehow to different genetic backgrounds leading to the occurrence of incomplete penetrance. Finally, this PEm trait expression appears to behave independently from environmental factors.

Conflict of interest

Authors declare no conflict of interest

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