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Doubled haploid ramets *via* embryogenesis of haploid tissue cultures

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ABSTRACT

Tissue culture in the oil palm business is generally concerned with the multiplication (clonal production) of dura, pisifera and tenera palms. These are all normal diploids (2n=2x=36). Sumatra Bioscience has pioneered haploid tissue culture of oil palm (n=x=18). Haploid oil palm is the first step in producing doubled haploid palms which in turn provide parental lines for F_1 hybrid production. Chromosome doubling is known to occur during embryogenesis in other haploid cultures, e.g. barley anther culture. Haploid tissue cultures in oil palm were therefore set up to investigate and exploit spontaneous chromosome doubling during embryogenesis. Flow cytometry of embryogenic tissue showed the presence of both haploid (n) and doubled haploid (2n) cells indicating spontaneous doubling. Completely doubled haploid ramets were regenerated suggesting that doubling occurred during the first mitoses of embryogenesis. This is the first report of doubled haploid production in oil palm via *haploid tissue culture. The method provides a means of producing a range of doubled haploids in oil palm from the 1,000 plus haploids available at Sumatra Bioscience, in addition the method also produced doubled haploid (and haploid) clones.*

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INTRODUCTION

Haploid is a general term used to describe an individual that has half a set of chromosomes (n). An individual with a complete set of chromosome pairs is described as diploid (2n). Oil palm is a diploid species (2n = 36). Nelson, et al. 2009 has described the production and identification of spontaneous haploids in oil palm seedlings (n = 18). Sumatra Bioscience (SumBio) now has a haploid germplasm collection of over 1,500 palms (Sitorus et al. 2010). The haploids in themselves have little value in breeding as they are sterile and therefore there is great interest in doubling the chromosomes of haploids to produced doubled haploids that are fertile and homozygous.

Haploid (H) plants are normally produced through sporophytic development of either the male or female gametic cells. The re-programming and redirected development of gametic cells to produce haploid and doubled haploid plants normally involves the tissue culture processes of anther culture or microspore culture (on the male side) or ovule culture (female side). Other methods include aberrant pollination such as wide crossing (using pollen from a distant species) (Dunwell et al. 2010). In some systems doubled haploids are produced spontaneously, especially if a embryogenesis system is involved (Cistué, et al, 2006, Szarejko, 2003). In others, chemicals such as colchicine are used to induce chromosome doubling in doubled haploid (DH) production.

Doubled haploid plants are completely homozygous as the doubling process duplicates the existing single set of chromosomes to produce identical chromosome pairs. Doubled haploidy provides a shortcut in the production of homozygous, true breeding lines that can be developed directly as cultivars. In Europe, it is estimated that 50% of contemporary barley cultivars are produced *via* a doubled haploid system. Other applications of doubled haploidy include the production of DH parents for F_1 hybrid seed production. F_1 hybrids exploit heterosis and have the capacity to produce greater yields than conventional cultivars; they are also uniform and stable and off high value (Dunwell, 2010). SumBio is developing haploid oil palm germplasm with the intention to producing DHs and thereby F_1 hybrids in oil palm, this represents a major breakthrough in oil palm breeding.

The culture of haploid tissues of oil palm offers potential in generating doubled haploids.

MATERIALS AND METHODS

SumBio started nursery planting of the haploid oil palms in 2006 and continues to do so, with the production of 30-50 new haploids per month (Sitorus et al. 2010). The first sampling of haploid oil palms for tissue culture began in 2008 by taking leaf samples from 1.5 years old palms in the nursery. A total of 80 haploids were sampled.

Haploid tissue culture from haploid oil palm leaf started with sampling leaf cylinder with length of 15 cm. A standard clone production method was used. Leaf tissues were extracted inside a laminar air flow bench and sterilized with 70% chlorox

for 20 minutes followed by 4 water rinses. Leaf segments (3 cm) were then cultured in liquid medium for one week incubation. One centimeter segments with the middle rib were then cultured on solid media. Explants and the callus cultures produced were maintained in a dark culture room at 25-30 ºC, with 8 weeks transfer intervals

RESULTS AND DISCUSSIONS

Leaf explants of haploid oil palm began to produce callus after 8 weeks incubation on solid media and continued until the third sub-culture. Twenty explants produced callus among total 80 leaf explant (25% callogenesis). The haploid ortet response was similar to diploid oil palm callogenesis which has an average of 24% callogenesis of explant set up.

Embryoid cultures were extracted after seven sub-cultures and continued up to 10 sub-cultures. In total there were five embryoid cultures produced that continued for embryoid multiplication and shoots production.

Figure 1. Embryogenic callus

Figure 2. Embryoid culture

Callus and embryoid cultures (Figures 1 and 2) were sampled for ploidy analysis using flow cytometery. The results showed there was no ploidy change up to and during callogenesis after 65 weeks (8 sub-cultures), with all cultures being 100% haploid. Ploidy-changes started to occur during embryogenesis, three weeks after setting up embryoid cultures: tissues sampled were composed of both haploid and diploid cells. This indicated that spontaneous doubling was associated with embryo production.

Regenerated shoots (at least 2 cm in length) were cultured on a root induction medium. There were 23 ramets harvested at two different times during the rooting stage (Table 1). Figure 3 shows the first ramets produced from haploid oil palm, which were sub-cultured onto rooting media containing NAA for two sub cultures, and these were progressed to hardening media (non-hormone media). Ploidy analysis was carried out by taking leaf samples of the youngest frond and tested using a flow cytometer as described by Nasution et al. (2010). Ploidy analysis was carried out twice for the first 12 ramets with an eight week transfer interval between first and

second sub-culturing. The result showed the presence of 6 haploids, 16 diploids and one mixoploid oil palm ramet (the later is believed to be in a transitional phase). These results show that haploid oil palm tissue culture can be exploited to induce spontaneous chromosome doubling in oil palm.

Table 1. Ramet ploidy analyses.

No.	Ortet	1st leaf sample	2nd leaf sample
1	H26. 01	2n	2n
2	H26. 02	2n	2n
3	H26. 03	2n	2n
4	H26. 04	2n	2n
5	H26. 05	n	n-2n
6	H26. 06	n	n
7	H26. 07	n	n
8	H26. 08	n	n
9	H26. 09	2n	2n
10	H26. 10	2n	2n
11	H26. 11	n	n
12	H26. 12	n	n
13	H26. 13	2n	na
14	H26. 14	2n	na
15	H26. 15	2n	na
16	H26. 16	2n	na
17	H26. 17	2n	na
18	H26. 18	2n	na
19	H26. 19	2n	na
20	H26. 20	2n	na
21	H26. 21	2n	na
22	H26. 22	n	na
23	H26. 23	2n	na

Figure 3. Haploid and doubled haploid ramets produced from a haploid ortet (on rooting media)

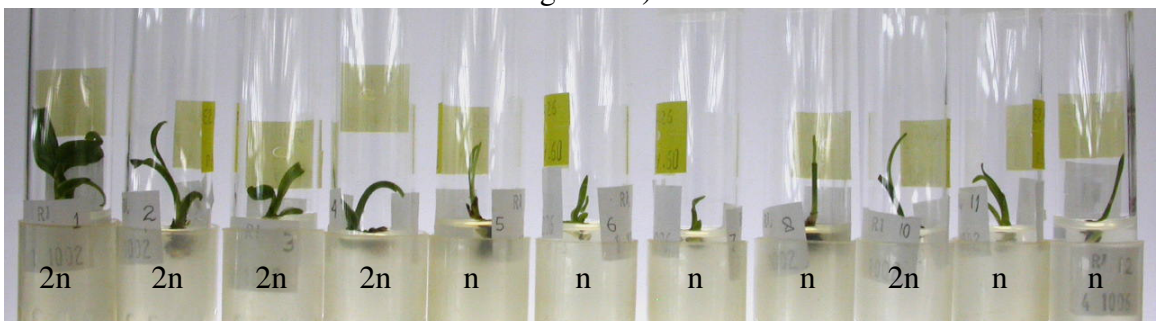

Figure 4. Growth of ramets after 8 weeks on rooting media

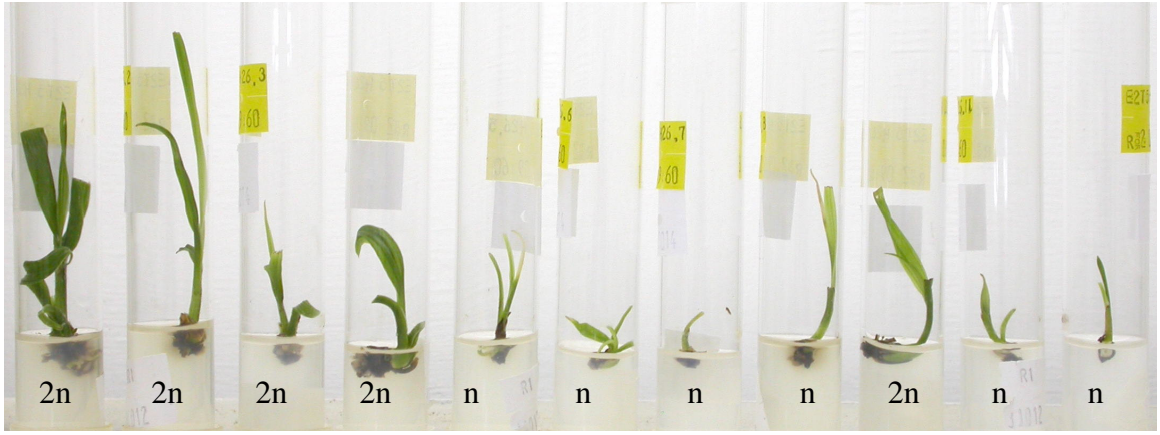

Figure 4 The growth of haploid and doubled haploid ramets after 8 weeks on rooting media. By comparing Figures 3 and 4 it is obvious that the double haploid oil palm ramets responded well to rooting media conditions whereas the haploids exhibit poor growth.

The doubled haploids and haploids, and the original ortet were tested for homozygosity using 60 SSR markers, no heterozygosity was found.

These results show that in vitro embryogenesis of haploid cultures of oil palm can be exploited to produce and clone doubled haploids. These are 100% homozygous and represent a valuable genetic resource for breeding and genetics.

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