HAPLOID INDUCTION OF KENAF (*HIBISCUS CANNABINUS* L.), OKRA (*ABELMOSCHUS ESCULENTUS* L.) AND SPRING ONION (*ALLIUM FISTULOSUM* L.) USING ANther, OVary AND OVule CULTURES

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Haploid Induction of Kenaf (*Hibiscus cannabinus* L.), Okra (*Abelmoschus esculentus* L.) and Spring Onion (*Allium fistulosum* L.) Using Anther, Ovary and Ovule Cultures

by

Ahmed Mahmood Ibrahim

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

Faculty of Agro Based Industry

UNIVERSITI MALAYSIA KELANTAN

2016
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>h</td>
<td>Hour</td>
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<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MS</td>
<td>Murashigae and Skoog</td>
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<td>N6-benzyladenine</td>
</tr>
<tr>
<td>GA</td>
<td>Gibberellic acid</td>
</tr>
<tr>
<td>HCl</td>
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<tr>
<td>IAA</td>
<td>Indoleacetic acid</td>
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PMC  Pollen mother cell
X  A change in the relative performance of a ‘< character » of two or more genotypes measured in two or more environments.
DH  Double haploid
RAPD  Random Amplified Polymorphic DNA
MS  Murashige and Skoog medium
N6  CHU N6 Basal Medium
MN6  Modified N6 medium
B5  Gamborg Medium
BDS  Modified B5
Penghasilan tumbuhan haploid daripada kenaf (Hibiscus cannabinus L.), bendi (Abelmoschus esculentus L.) dan daun bawang (Allium fistulosum L.) menggunakan kultur anter, ovari dan ovul

ABSTRAK

Penghasilan tumbuhan haploid daripada kultur anter dan ovari yang diikuti oleh kromosom ganda dua boleh menghasilkan baris induk homozigot dalam masa yang lebih singkat berbanding dengan penghasilan baris biakbaka dalam (inbred) dengan kaedah konvensional melalui cacauan sendiri berulang-ulang. Tesis ini menerangkan kajian yang dijalankan untuk mengkaji potensi kultur anter, mikrospora (debunga), ovari dan ovul daun, kenaf (Hibiscus cannabinus L.), bendi (Abelmoschus esculentus L.) dan bawang (Allium fistulosum L.) untuk penghasilan tumbuhan haploid. Anter, ovari dan ovul diambil dariedan bunga pada peringkat berbeza dan kebolehan untuk menghasilkan kalus haploid atau embriogenesis somatik dan seterusnya menjana semula kepada tumbuhan haploid dikaji. Untuk tujuan tersebut, beberapa faktor seperti masa permulaan bunga dan pengumpulan tunas bunga, jenis media, kepekatan dan kombinasi hormon, kepekatan sukrosa dan keadaan kultur telah dikaji. Tunas bunga dengan ukuran berbeza telah diseksi untuk menentukan tahap perkembangan sebelum digunakan dalam pelbagai prarawatan (sejuk dan kolkisina) dan kemudian anter, mikrospora, ovari dan ovul telah dikulturkan ke dalam kombinasi hormon yang berbeza (NAA, IAA, 2,4-D, KIN, BAP, IBA, ZTN, 2iP dan TDZ) dan berlainsen kepekatan. Kultur ini telah diinkubasi dalam keadaan gelap dan terang. Peringkat perkembangan mikrospora terbaik untuk penginduksian kalus telah diperolehi daripada 8 mm tunas bunga bagi kenaf dan 12 mm tunas bunga bagi bendi dari kemunculan kelompok bunga pertama. Manakala peringkat perkembangan terbaik bagi ovari dan ovul adalah satu atau dua hari sebelum anthesis bagi kenaf dan bendi, dan 3-5 mm tunas bunga bagi daun bawang. Kalus haploid dan akar dapat dihasilkan daripada anter, ovari dan ovul bagi kenaf dan bendi. Penjanaan semula planlet haploid boleh diperolehi oleh daun bawang menggunakan kultur bunga dan ovari yang telah disahkan oleh kajian ploidi menggunakan aliran sitometri. Hasil kajian menunjukkan kesar masa permulaan bunga adalah antara fakor penting bagi kultur anter dan ovari. Tiada perbezaan yang signifikan dalam peratusan penginduksian kalus bagi prarawatan sejuk, 0.5 mg/l TDZ atau 3.0 mg/l BAP dicampur dengan 2.0 mg/l NAA menghasilkan peratusan penginduksian kalus yang tertinggi (95%). Antara tiga media penginduksian, media MS adalah media yang terbaik dengan purata penginduksian kalus sebanyak 95%. Perbezaan yang signifikan telah diperhatikan dalam penginduksian kalus dengan kepekatan sukrosa sebanyak 3%. Penyimpanan di dalam tempat gelap selama 28 hari menghasilkan peratusan penginduksian kalus dan akar paling tinggi (92.5%). Tiada pucuk dapat dihasilkan daripada kenaf dan bendi walaupun selepas beberapa rawatan dan subkultur lanjutan. Tesis ini mewujudkan titik perlu untuk penelitian bagi tiga tanaman ini. Protokol yang dihasilkan untuk penghasilan planlet haploid dalam daun bawang boleh membantu dalam program pembiakan bagi peningkatan trait genetik daripada daun bawang.
ABSTRACT

The production of haploid plants by anther and ovary cultures followed by chromosome doubling can produce homozygous parent lines in a relatively shorter time compared to the production of inbred lines by conventional method through repeated selfings. The thesis describes the studies undertaken to investigate the potential of anther, microspores (pollens), ovary and ovule cultures of kenaf (Hibiscus cannabinus L.), okra (Abelmoschus esculentus L.) and spring onion (Allium fistulosum L.) for the production of haploid plants. Anther, ovary and ovule were excised from flower buds at different stages. The ability to produce haploid callus or somatic embryogenesis and thereby regenerate into haploid plants were investigated. Several factors such as flower bud initiation time, type of media, plant growth regulator (PGR) combinations and concentration, sucrose concentration and dark periods have been evaluated. The flower buds of different sizes were dissected to determine their stage of development before subjected to various pretreatments (cold and colchicines) and then the anthers, microspores, ovaries and ovules were cultured on different PGR combinations (NAA, IAA, 2,4-D, KIN, BAP, IBA, ZTN, 2iP and TDZ) and concentrations. The cultures were incubated in both dark and light condition. The suitable developmental stage of microspore for callus induction was obtained from 8 mm length of flower buds in kenaf and 12 mm length of flower bud in okra from the first batch flower emergence and 2 mm length flower bud in spring onion. While the suitable developmental stage for ovaries and ovules were one or two days before anthesis of kenaf and okra and and 3-5 mm flower bud in spring onion. Haploid calli and root were produced from the anther, ovary and ovule of kenaf and okra. Regeneration of haploid plantlets could be obtained in spring onion using flower and ovary cultures which were confirmed by ploidy test using a flow cytometry. The results of the study revealed that the effect of flower bud initiation time was an important factor in anther and ovary cultures. There were no significant difference in percentage of callus induction on cold pre treatment, 0.5 mg/l TDZ or 3.0 mg/l BAP combined with 2.0 mg/l NAA gave highest percentage (95%) of callus induction. Among the three callus induction media, MS medium was the most responsive medium with an average of 95% callus induction. A significant differences were observed at 3% of sucrose concentration on callus induction. Incubation in a dark place for 28 days in dark place gave highest percentage (92.5%) of callus and root induction. No shoot was developed from kenaf and okra despite several treatments and further sub-culturing. The study can be starting point for the improvement of the three crops. The protocols developed for the production of haploid plantlets in spring onion helpful in a breeding program for the improvement of genetic traits of spring onion.
1.1 Importance of haploid

Haploids are sporophytic plants that contain the gametic chromosome number. Haploids arise from diploid species containing a single genome are described as monoploids. Haploids derived from polyploid species, containing two or more genomes are called polyhaploids. Haploid plants become doubled haploids (DHs) as a result of chromosome doubling. The doubled-haploid methodology offers several advantages to plant improvement programs as it can facilitate a rapid approach to homozygosity.

Haploid plants are of great interest to geneticists and plant breeders as they offer the opportunity to examine genes in the hemizygous condition and facilitate identification of new mutations. Plant breeders value haploids as a source of homozygosity following chromosome doubling from which efficient selection of both quantitative and qualitative traits can be accomplished. Since haploid plants carry only one set of alleles at each locus, homozygous and homogeneous lines can be achieved upon doubling. This method can be applied for evaluation of qualitative and quantitative traits, avoiding the masking of recessive genes. The evaluation of possible environment x genotype interactions, and identification of superior parental combinations can also be done properly.
Other benefits include detection of genetic linkages; determination of recombination values (Snape, 1988) and molecular genome identification.

The production of F1 hybrids is considered as one of the main goals in crops breeding program. The main restriction to achieve it is the length of time needed to produce homozygous parental materials. The most time-consuming and work-intensive method through the conventional breeding process is troublesome as it requires manual self-pollination to generate pure homozygous parent lines. Eight or more generations of inbreeding are needed to establish homozygous lines that can be applied in hybrid production. This process can be enhanced by using doubled haploid (DH) lines as components of hybrid cultivars.

1.2 Kenaf

Kenaf (*Hibiscus cannabinus* L.) belongs to the Malvaceae family, under the section Furcaria that is closely related to cotton, okra, hollyhock and roselle. Kenaf is an annual fiber crop cultivated for numerous uses such as for paper pulp, fabrics, textile, building materials, biocomposites, bedding material, oil absorbents and many more (Andrea & Efthimia, 2013). Nowadays, it has been cultivated in more than 20 countries worldwide. However, this plant is considered as new in Malaysia and is cultivated to replace tobacco plantation, which is no longer supported by the government (Roslan *et al.*, 2011). Kenaf can grow fast and achieves 5 to 6 m in height and 2.5 to 3.5 cm in diameter.
within 5 to 6 months. Kenaf has a unique combination of long bast and short core fibers which makes it suitable for a range of paper and cardboard products. Fifty five percentage of dried kenaf stalks are used to make paper while the waste from the process can be utilized for fertilizer and feed binder. Home gardens grown kenaf usually have more tender upper leaves and shoots which are eaten either as raw or cooked food (Gordon 1994).

The National Kenaf and Tobacco Board (LKTN) contrive the development of kenaf cultivation in order to replace the current tobacco cultivation in Kelantan. Moreover, the Malaysian government also emphasizes in diversifying and commercializing the downstream kenaf based industries including the pulp and paper industry in cooperation with the private sectors. However, the cultivation of kenaf is not attractive to the farmers because the income from kenaf yields is lower than that of tobacco. The low profit gained from kenaf compared to tobacco makes kenaf unpopular among the farmers. The low yields of kenaf is due to lack of superior characteristics such as small diameter stem, short plant height and early flowering resulting in less fiber yield. Therefore, development of superior variety with better agronomic traits is highly needed. The establishment of protocols for haploid and double haploid lines could accelerate the breeding program for the development of the improved kenaf cultivar.