ABSTRACT

The present work with Annona species was carried out with three main objectives: (i) To develop methods for clonal propagation of the hybrid and its parents. A comparative study was also conducted with the parents Annona squamosa and Annona cherimola and their hybrid to find out if there were any differences with regard to their hormonal and other requirements. (ii) For production of haploids from Annona squamosa using the technique of another culture and (iii) To obtain triploid callus from cultured endosperm of Annona squamosa with a view to obtaining triploid plants.

These studies have been divided into four main chapters. The results obtained in chapters III, IV, V, and VI have been summarised as follows:

CHAPTER III: Studies on multiple shoot induction from seedling explants and differentiation of stem-derived callus of Annona squamosa

These studies on shoot induction from seedling stem and leaf explants and stem-derived callus of A. squamosa were conducted with respect to the hormonal and other growth requirements.

i) Of the various seedling explants used, the hypocotyl segments followed by stem, leaf, and shoot apex were the best explants.

ii) Of the various basal media tested, the best response was obtained on MS.

iii) Two to four percent sucrose was found optimum.

iv) The optimum temperature was 27°C and light intensity 1000 lux.

v) Maximum number of shoots formed from stem and stem callus on MS basal medium supplemented with 0.2 mg/l Kin and 0.5 mg/l BAP, while for leaf explants 0.5 mg/l Kin and 2.0 mg/l BAP was found best.

vi) Inclusion of auxins in MS-1 or MS-2 medium decreased the number of shoots from stem and leaf explants and almost completely inhibited shoot regeneration from callus explants.

vii) All complex extracts tested (CH, CM etc.) slightly inhibited shoot formation from stem and leaf explants. Regeneration of plants from stem callus was greatly enhanced in the presence of 0.05% CH.
As a result of these experiments the best medium for obtaining maximum shoot proliferation was MS basal medium supplemented with 0.2 mg/l Kin and 0.5 mg/l BAP for stem explants; MS basal medium with 0.5 mg/l Kin and 2.0 mg/l BAP for leaf and 0.2 mg/l Kin, 0.5 mg/l BAP and 0.05% CH for stem callus differentiation.

The most responsive region of the leaf for maximum induction of shoots using MS-3 medium was the basal portion of the leaf containing the petiole followed by the region of the leaf containing the leaf base without the petiole and the petiolar region without lamina.

Histological Studies

Stem and leaf explants grown in culture were histologically examined in order to confirm the origin of the shoot buds. These studies revealed the hypodermal (cortical) origin of shoot buds in stem explants. In leaf the buds arose by the meristematic activity of both epidermal and hypodermal (mesophyll) cells.

CHAPTER IV: Comparative studies on plantlet regeneration from mature tree explants of Annona hybrid and its parents A squamosa and A cherimola

Studies on shoot regeneration

This section describes the culture requirements to obtain maximum shoot bud induction and elongation from nodal explants of the hybrid Annona and its parents. A comparison of the culture requirements of the hybrid with that of its parents has also been made. The following points are relevant in this connection.

i) A MS basal medium supplemented with 0.5 mg/l Kin, 0.5 mg/l BAP and 0.01% CH (MS-5) was found to be the best on which opening of maximum number of cultured buds took place. In Annona hybrid and A squamosa 50% of the buds opened while with A cherimola the response was 70%. The average number of shoots per explant being 4 and 5 respectively.

ii) BAP followed by Kin was responsible for maximum shoot bud proliferation in the hybrid as well as the parent cultures.

iii) A combination of Kin and BAP was superior producing the highest number of shoot buds compared to other cytokinin combinations. However, the number of shoot buds per tube was highest in A cherimola (13-16 buds) and only 5 - 7 shoot buds in A squamosa and the hybrid.
iv) CH was found to be superior to other complex additives like CM, YE, ME and Ed, and was optimum at 0.01% in all three species.

v) Addition of auxins to the medium initiated the growth of callus with a depression in shoot numbers in all three species.

vi) 30°C was found most suitable for Annona hybrid, 27°C for A. squamosa, and 25 and 27°C for A. cherimola.

vii) A light intensity of 800 - 1200 lux was found optimum for Annona hybrid, while 1200 lux supported maximum growth of shoots in A. cherimola and A. squamosa.

From the above experiments it was concluded that the medium favourable for maximum proliferation of shoot buds from nodal explants was MS agar medium supplemented with 0.5 mg/l Kin, 2.0 mg/l BAP and 0.01 CH for the hybrid as well as its parents.

viii) Liquid medium proved deleterious for all the species.

ix) For elongation of the shoot buds a medium supplemented with lower cytokinin levels was found efficacious both for the hybrid and the parents viz. Kin (0.05 mg/l) combined with BAP (0.1 mg/l) for elongation of Annona hybrid and A. cherimola shoot buds while Kin (0.02 mg/l) and BAP (0.3 mg/l) for shoot buds in A squamosa.

From the studies it was clear that the hybrid Annona differed from its parents A squamosa and A cherimola in the following respects:

(a) In the absence of cytokinins no shoot bud induction was seen in the hybrid while 1 - 3 buds were produced in the parent cultures.

(b) At 0.1% CH there was a total inhibition of shoot buds in the hybrid while 1 - 3 buds were found in the cultures of A squamosa and A cherimola. Maximum shoot bud formation was seen at two concentrations of CH i.e. 0.005 and 0.01%. In the parents only CH at 0.01% was responsible for producing the highest number of shoot buds.

(c) In the presence of the auxins NAA and IPA shoot bud formation was suppressed in Annona hybrid although varying numbers of shoot buds were produced in the parent cultures.

(d) 2, 4-D and IPA were responsible for callus formation in the hybrid unlike the parent cultures which callused with 2, 4-D and NAA.

(e) In the hybrid maximum number of shoot buds occurred at a temperature of 30°C, in A cherimola at 25 and 27°C, and for A squamosa at 27°C.
Studies on root regeneration from seedling (A squamosa) and mature tree shoots

This section deals with conditions required for rooting the regenerated shoots obtained from both seedling (A squamosa) and mature tree shoots. Studies on root regeneration emphasize the following requirements for successful rooting of cultured shoots:

Seedling (A squamosa)

(i) Of all the auxins tested, IBA was found the most effective one.
(ii) Treatment with 20 mg/l IBA in MS (1/2 strength) liquid medium for 72 h in dark before transfer to an auxin-free medium was found necessary.
(iii) The auxin-free medium consisted of a different basal salt composition i.e. Anderson medium, than the one used for treatment of shoots with IBA.
(iv) Presence of 0.25% activated charcoal in the auxin-free Anderson medium was necessary for root initiation which took place within 25 - 30 days on this medium.
(v) Reduction in the salt concentration in Anderson medium to half resulted in an increase in the rooting percentage from 10 to 20.
(vi) A mixture of auxins and also use of phloroglucinol for rooting were found ineffective in raising the percentage of rooted shoots.
(vii) Rooted seedling shoots required subsequent transfer from charcoal medium to Anderson (1/2 strength) liquid medium for further growth of plantlets before transfer to pots containing soil.

Mature tree shoots

The treatments for rooting of seedlings was not found successful for root initiation from shoots of mature trees. The following modifications ad to be carried out:

(i) Treatment with higher IBA concentration (50 mg/l) for longer periods (16 days).
(ii) The use of a semisolid MS (1/2 strength) medium for the treatment of shoots was found superior than the same medium in the liquid state.
(iii) An initial dark period of 3 days followed by 13 days light (1000 lux) was found to increase the percentage of rooting from 5 to 43% in the case of Annona hybrid and from 5 to 20% for A cherimola. In A squamosa this treatment did not enhance the rooting response, which remained low (5%).
(iv) Following the auxin treatment, transfer of shoots to Anderson (1/2 strength) semisolid medium lacking auxin but with 0.25% activated charcoal
was necessary for root initiation. This stage is common with the rooting procedure used for seedling shoots.

Transfer to field

60% of the regenerated plants (seedling and mature tree shoots) survived in field.

CHAPTER V: Studies on anther culture of Annona squamosa

This chapter describes various culture conditions for obtaining haploid plants through anther culture of Annona squamosa. These studies highlight several important conditions which have to be fulfilled for obtaining success with cultured anthers in this species:

(i) Culture of anther groups was necessary in comparison to single anthers.
(ii) Dissection of the flower buds in a 0.25% activated charcoal solution prior to inoculation in a medium lacking activated charcoal proved effective.
(iii) The most responsive bud size was found to be between 10 and 15 mm. These contained pollen grains in the early uninucleate to mid-uninucleate stage.
(iv) Cold or high temperature pretreatments to the flower buds proved deleterious for anther culture, because the cultured anthers turned brown and died within 20 days on the medium.
(v) An initial 7 days incubation in the dark followed by light conditions (18 h, 1000 lux) was found most suitable for the highest percentage of responding anther groups.

Hormonal requirements for anther culture were systematically carried out and the results are summarized below.

(i) Of the different basal media tested, Nitsch medium was found best followed by N6 medium.
(ii) The presence of 800 mg/l glutamine, 100 mg/l serine and 1000 mg/l inositol in the medium used for inoculation of fresh anther groups was found effective for anther swelling. The response was poor when any of the above compounds was eliminated from the culture medium.
(iii) A three component hormone supplement comprising IAA (2 mg/l), 2,4-D (3 mg/l) and Kin (0.5 mg/l) was found to be most effective for the maximum number of swollen anther groups.
(iv) A sucrose level of 6% in the medium was found optimum for the initial swelling of the anther groups. Bursting of the swollen anthers and callus production occurred on transfer to a Nitsch basal medium containing 5 mg/l IAA and a lowered sucrose concentration of 2%.
Anther callus thus obtained could be subcultured on the same medium (N+ 5 mg/l IAA), without any loss in the capacity for callus proliferation. Growth pattern of the anther callus showed the maximum growth between 25 - 30 days of culture.

Differentiation studies carried out on anther callus showed that BAP (2 mg/l) with 1 mg/l NAA could induce complete plantlets in 10% of the cultures while 2 mg/l BAP in combination with 0.1 mg/l IAA resulted in multiple shoot formation in 40% of the cultures.

Root tip squashes of the regenerated plantlets revealed its haploid nature (n = 7). Attempts to transfer the haploid plantlets to field have so far not met with success.

CHAPTER VI: Studies on endosperm culture of Annona squamosa

This chapter describes the hormonal and physical conditions of the culture medium for obtaining maximum callus proliferation from cultured endosperm of Annona squamosa. Some experiments to induce differentiation of the callus are also described.

Studies were initially carried out with immature endosperm tissue obtained from young green fruits of Annona squamosa before the development of a hard seed coat. The seeds were inoculated either intact or after splitting them into two halves. Callus proliferation, however was not observed in these cultures.

Studies were then conducted using endosperm tissue obtained from mature seeds of Annona squamosa. The following are the conditions for successful culture of endosperm based on various experiments:

(i) The source of inoculum used was germinated seed (2 - 4 days after radicle emergence) from which the endosperm (without the embryo) was cultured keeping the cut surface touching the medium.

(ii) Of the different basal media tested White's basal medium was found to be most effective followed by N6.

(iii) Addition of complex additives like CH, CM, YE, ME and Ed were not useful for enhancing callus production in cultured endosperm.

(iv) A combination of two cytokinins, Kin (0.1 mg/l) and BAP (0.2 mg/l) was found the most suitable.

(v) Of all the auxins tested NAA at 1 mg/l was responsible for maximum callus proliferation followed by 2, 4-D and IAA.
(vi) GA$_3$ was also essential for the proliferation of endosperm callus and its optimum concentration was found to be 1 mg/l.

(vii) The optimum level of sucrose was found to be 3-4%. There was no callus formation on a medium lacking sucrose.

(viii) A temperature of 27°C and a light intensity of 1000 lux was best for growth of callus.

Endosperm callus could be periodically subcultured and so far even after 10 subcultures there has been no reduction in the capacity to form callus. The growth pattern of endosperm callus showed its maximum growth between 40 and 50 days in culture followed by a decline in growth.

Cytology of the endosperm callus (at third subculture) revealed its triploid nature (3 n = 21).

Differentiation studies

Various media compositions were tried to obtain differentiation in endosperm callus. Organogenesis, however, was limited only to tracheid differentiation observed in callus cultured on a Nitsch basal medium supplemented with 2 mg/l BAP and 0.1 mg/l IAA and differentiation of roots in 5% callus masses in the same basal medium containing 5 mg/l IAA. Shoot differentiation has not been obtained.

From all the studies carried out with Annona species certain useful conclusions can be drawn.

1. Tissue culture techniques can be used for propagation of Annona hybrid which is difficult to propagate by conventional methods.

2. Haploid plants produced via the anther culture technique can be of immense importance for mutant isolation and production of homozygous lines for breeding programmes of this highly heterozygous fruit tree.

3. Endosperm plants if produced could be very significant for obtaining seedless fruits and also in improving fruit quality and in the production of more vigorous and fast-growing trees of this fruit species.