IN VITRO CULTURE STUDIES IN FOUR DIOECIOUS VARIETIES OF CARICA PAPAYA L. USING AXILLARY BUDS FROM FIELD-GROWN PLANTS

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Abstract: In vitro culture study via multiple shoots was attempted using axillary bud explants from field grown plants in four dioecious varieties of Carica papaya L. viz. Co-5, Madhur, Pusa Dwarf and Washington. Established axillary bud explants were inoculated on various induction and proliferation media for the multiple shoots and the elongation media for the elongation of the shoots. IBA dip treatment given to the cut end of the shoots produced healthy roots. The combination of soil: vermiculite: cowdung (1: 1: 1) were used as substratum for the hardening of the plantlets.

Key words: In vitro culture, Carica papaya L., Axillary buds.

INTRODUCTION

Carica papaya L. varieties Co-5, Madhur, Pusa Dwarf and Washington were selected for the present study. These dioecious varieties differed in traits such as tree size, fruit size, colour, flavour and its suitability for table purpose or latex extraction. Carica is mostly dioecious with a strong dimorphism between staminate and pistillate flowers (Ronse Decraene and Smets, 1999). The variation in sex and detection of sex only after flowering made Carica papaya L. an interesting system for study. The main objective of the present work was to develop a protocol for the clonal propagation of dioecious varieties of Carica papaya L. via multiple shoot from the axillary bud explants of the field grown plants.

MATERIALS AND METHODS

In the present study field grown plants after flowering were selected. Axillary bud explants from male and female plants were maintained separately. Pretreated axillary bud explants were given pulses of GA₃ (500 mg/l) for 1 hour and then inoculated on MS medium (Murashige and Skoog, 1962) fortified with MgSO₄ (400 mg/l) for establishment. The established axillary bud explants for shoot bud initiation were inoculated on various induction media such as basal MS medium, MS medium fortified singly with IAA or NAA (0.05, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l), BAP, KIN or AS (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) or AS at a higher range of (10, 20, 40, 60, 80 and 100 mg/l); MS medium fortified with various concentrations of auxins or cytokinins in different combinations: BAP + NAA; BAP + IAA; KIN + NAA; KIN + IAA; AS + NAA; AS + IAA and BAP + NAA + AS; BAP + IAA + AS; KIN + NAA + AS; KIN + IAA + AS; combinations of cytokinins: BAP + KIN; BAP + AS; KIN + AS and combinations of auxins: NAA + IAA also MS medium fortified with two cytokinins and one auxin.

Axillary bud explants were then inoculated on various proliferation media i.e. MS medium (1-10% sucrose) fortified with BAP (5 mg/l), AS (10 mg/l) and NAA (2 mg/l) for proliferation of the shoot buds. Multiple shoot buds of same length (1.0 cm) were later separated and inoculated on various elongation media such as MS, MS+B5 and ½ MS media, MS medium fortified with GA₃, IAA and NAA (0.5, 1.0,
plants. In the induction media, AS (0.5 - 5.0 mg/l) did not give rise to the initiation of multiple shoots and AS (20 - 100 mg/l) gave rise to stress symptoms. In Carica papaya L. varieties Co-5, Madhur, Pusa Dwarf and Washington the axillary bud explants inoculated in the induction medium i.e. MS medium fortified with BAP (5.0 mg/l), AS (10 mg/l) and NAA (2.0 mg/l) gave rise to 1.93, 1.81, 1.38 and 2.15 shoot buds respectively and the axillary bud explants inoculated in the proliferation medium i.e. MS medium (6% sucrose) fortified with BAP (5.0 mg/l), AS (10 mg/l) and NAA (2.0 mg/l) gave rise to 6.16, 5.90, 4.14 and 8.30 shoot buds respectively (Table 1). The shoot buds failed to elongate when inoculated on MS, MS+B5, ½ MS media and proliferation medium supplemented with CM (5%) or sucrose (0-3% and 7-10%); MS medium fortified with GA₃, IAA or NAA and proliferation medium supplemented with CM (15%) induced excessive callusing at the base of the shoot buds. Proliferation medium supplemented with CH, U and Tri promoted elongation however it also resulted in fragile, yellowing and shedding of leaves thus they were not used for further study. However, proliferation medium supplemented with CM (10%) promoted elongation. In 8 weeks the length of the shoots was 2.76, 2.70, 2.26 and 2.83 cm in Carica papaya L. varieties Co-5, Madhur, Pusa Dwarf and Washington respectively (Table 1). Rooting was not observed in shoots inoculated on MS medium fortified with NAA (1.0-5.0 mg/l) or base of the shoot dipped in NAA, IAA or IBA (250-3000 mg/l) for 10 and 20 seconds and inoculated on MS medium. Profuse callusing was observed at the base of the shoots, which were dipped in NAA and IAA.

RESULTS AND DISCUSSION

Initiation of the multiple shoot buds did not take place when axillary bud explants were inoculated on basal MS medium, MS medium fortified singly with various concentrations of IAA or NAA, BAP or KIN and AS in two ranges; MS medium fortified with auxins (0.05- 0.5 mg/l) and cytokinins (0.5 - 3.0 mg/l) in various combinations. MS medium fortified with auxins (3.0 - 5.0 mg/l) and cytokinins (0.5 - 3.0 mg/l) in various combinations resulted in excessive callus formation. MS medium fortified with BAP, AS and IAA; KIN, AS and NAA or KIN, AS and IAA gave rise to the initiation of the shoot buds with excessive development of callus at the base of the axillary bud explants. In the induction media, AS (0.5 - 5.0 mg/l) gave rise to the initiation of the shoot buds with excessive development of callus at the base of the axillary bud explants.

Table 1: Response of axillary bud explants in Carica papaya L. varieties Co-5, Madhur, Pusa Dwarf and Washington inoculated on different media for the initiation, proliferation and elongation of the shoot buds.

<table>
<thead>
<tr>
<th>Varieties</th>
<th>Shoot bud numbers</th>
<th>Shoot bud numbers</th>
<th>Shoot bud numbers</th>
<th>Shoot bud numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Varieties</strong></td>
<td><strong>Co-5</strong></td>
<td><strong>MA</strong></td>
<td><strong>PD</strong></td>
<td><strong>W</strong></td>
</tr>
<tr>
<td>Induction media</td>
<td>1.36±0.01</td>
<td>0.93±0.00</td>
<td>2.14±0.00</td>
<td>1.02±0.00</td>
</tr>
<tr>
<td>BAP + AS + NAA (mg/l)</td>
<td>1.02±0.00</td>
<td>0.52±0.05</td>
<td>1.48±0.01</td>
<td>0.83±0.00</td>
</tr>
<tr>
<td>Proliferation media</td>
<td>1.62±0.00</td>
<td>1.10±0.00</td>
<td>1.45±0.01</td>
<td>0.80±0.00</td>
</tr>
<tr>
<td>BAP + AS + NAA + Sucrose (6%)</td>
<td>1.02±0.00</td>
<td>0.25±0.00</td>
<td>0.26±0.00</td>
<td>0.50±0.00</td>
</tr>
<tr>
<td>Elongation media</td>
<td>1.02±0.00</td>
<td>0.93±0.00</td>
<td>1.02±0.00</td>
<td>0.83±0.00</td>
</tr>
</tbody>
</table>

Values are mean of three sets of determinants. Each set containing 10 explants. Abbreviations used in table and text: BAP: 6-Benzyleaminopurine; AS: Adenine sulphate; NAA: α-Naphthaleneacetic acid; Co-5: Coimbatore-5 variety; MA: Madhur variety; PD: Pusa Dwarf variety; W: Washington variety; MS: Murashige and Skoog medium (1962); MS + B₃: Macro and micro salts of MS medium and vitamins of B₃ medium; GA₃: Gibberelic acid; KIN: Kinetin; IAA: Indole-3-acetic acid.

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(250-3000 mg/l) for 30 seconds and inoculated on MS medium. The shoots inoculated on MS medium fortified with IAA or IBA (1.0-5.0 mg/l) produced roots however % rooting was low and time taken for rooting was more, thus these rooting media were not used for further study. The base of the shoot dipped in IBA (2500 mg/l) for 30 seconds, inoculated on MS basal medium showed 90.3, 89.3, 64.6 and 91.0 % rooting in 15, 16.6, 16 and 14 days in *Carica papaya* L. varieties Co-5, Madhur, Pusa Dwarf and Washington respectively. In the green house combination of soil: vermiculite: cowdung (1:1:1) used as a substratum could show 42, 52, 16 and 50 % survival of plantlets in *Carica papaya* L. varieties Co-5, Madhur, Pusa Dwarf and Washington respectively in 4 weeks (Table 2).

In the present study the axillary bud explants (Fig. A) of *Carica papaya* L. varieties Co-5, Madhur, Pusa Dwarf and Washington inoculated on MS medium (6% sucrose) fortified with BAP (5.0 mg/l), AS (10 mg/l) and NAA (2.0 mg/l) showed shoot bud initiation and proliferation. Proliferation media supplemented with CM (10%) promoted the elongation of the multiple shoots (Fig. B). *In vitro* culture of *Carica papaya* L. cv. ‘Rajshahi-red’ was initiated by culturing shoot apices from mature plant onto MS medium supplemented with 2ip (10 mg/l) + NAA (1.0 mg/l) and multiple lateral bud proliferation was induced after transferring into half the strength of MS medium (4 weeks) having BA (0.1 mg/l) + NAA (0.02 mg/l) (Hossain et al., 1991). In the present study root initiation after dip treatment in IBA (2500 mg/l) for 30 seconds inoculated on MS basal medium showed

<table>
<thead>
<tr>
<th>Rooting and hardening</th>
<th>Varieties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Co-5</td>
</tr>
<tr>
<td>Base of the shoot dipped in IBA (2500 mg/l) for 30 seconds, inoculated on MS basal medium</td>
<td>Rooting (%)</td>
</tr>
<tr>
<td>Time taken (d)</td>
<td>15 ± 1.00</td>
</tr>
<tr>
<td>Substratum - Soil: vermiculite: cow-dung (1:1:1) in green house in 4 weeks</td>
<td>Hardening success (%)</td>
</tr>
</tbody>
</table>

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![Fig. A: Carica papaya L. variety Co-5-axillary bud.](image1)

![Fig. B: Carica papaya L. variety Madhur - multiple shoots.](image2)

![Fig. C: Carica papaya L. variety Pusa dwarf – rooting.](image3)

![Fig. D and Fig. E: Carica papaya L. variety Washington–Hardening in green house and sfield.](image4)
maximum rooting (Fig. C). These plantlets were transferred to soil: vermiculite: cowdung (1: 1: 1) in the hardening chamber, green house and finally to the field (Fig. D and Fig. E). In dioecious variety (MF-1), IBA dip treatment given to cut end of shoot produced healthy root system within a month. A successful in vitro to in vivo transfer of 100% plantlets were achieved in substrate mix of Vermiculite: Celrich: Red soil: Peat and Perlite in ratio of 15: 50: 15: 15 (Bankar and Sharon, 1996).

Carica papaya L. is an excellent example of a crop that is extremely vulnerable, but economically very important. Vegetative propagation methods do not exist for large-scale plantations, hence clonal propagation through tissue culture is much desired. Economically important Carica papaya L. cultivars Co-5, Pusa Dwarf and Washington could be successfully regenerated in vitro by somatic embryogenesis (Saha et al., 2004). In the present study an attempt has been made to develop a protocol for the clonal propagation of dioecious varieties of Carica papaya L. through organogenesis by the direct multiple shoot formation from the axillary bud explants of the field grown plants.

REFERENCES

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Axillary bud explants were then inoculated on various proliferation media i.e. MS medium (1-10% sucrose) fortified with BAP (5 mg/l), AS (10 mg/l) and NAA (2 mg/l) for proliferation of the shoot buds. Multiple shoot buds of same length (1.0 cm) were later separated and inoculated on various elongation media such as MS, MS+B5 and ½ MS media, MS medium fortified with GA₃, IAA and NAA (0.5, 1.0,
2.0, 3.0, 4.0 and 5.0 mg/l), proliferation media supplemented with coconut milk [CM (5, 10 and 15%)], casein hydrolysate [CH (50 and 100 mg/l)], urea [U (0.1 and 0.5 mg/l)], triacontenol [Tri (1.0 and 2.0 mg/l)] or sucrose (0-10%) for elongation. Shoot buds (4-5 cm) with leaves (6-7 numbers) were later inoculated on MS medium fortified with various concentrations of NAA, IAA and IBA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) singly or the base of the shoot was dipped in various concentrations of NAA, IAA or IBA (250, 500, 1500, 2000, 2500 and 3000 mg/l) for 10, 20 or 30 seconds and then inoculated on MS basal medium for rooting. The plantlets were maintained in the hardening chamber and in green house on soil: vermiculite; cow-dung (1:1:1) for 4 weeks respectively. The plants were kept in shade house and gradually transferred to the field.

RESULTS AND DISCUSSION

Initiation of the multiple shoot buds did not take place when axillary bud explants were inoculated on basal MS medium, MS medium fortified singly with various concentrations of IAA or NAA, BAP or KIN and AS in two ranges; MS medium fortified with auxins (0.05-0.5 mg/l) and cytokinins (0.5 - 3.0 mg/l) in various combinations. MS medium fortified with auxins (3.0 - 5.0 mg/l) and cytokinins (0.5 - 3.0 mg/l) in various combinations resulted in excessive callus formation. MS medium fortified with BAP, AS and IAA; KIN, AS and NAA or KIN, AS and IAA gave rise to the initiation of the shoot buds with excessive development of callus at the base of the axillary bud explants. In the induction media, AS (0.5 - 5.0 mg/l) did not give rise to the initiation of multiple shoots and AS (20 - 100 mg/l) gave rise to stress symptoms. In *Carica papaya* L. varieties Co-5, Madhur, Pusa Dwarf and Washington the axillary bud explants inoculated in the induction medium i.e. MS medium fortified with BAP (5.0 mg/l), AS (10 mg/l) and NAA (2.0 mg/l) gave rise to 1.93, 1.81, 1.38 and 2.15 shoot buds respectively and the axillary bud explants inoculated in the proliferation medium i.e. MS medium (6% sucrose) fortified with BAP (5.0 mg/l), AS (10 mg/l) and NAA (2.0 mg/l) gave rise to 6.16, 5.90, 4.14 and 8.30 shoot buds respectively (Table 1). The shoot buds failed to elongate when inoculated on MS, MS+B5, ½ MS medium and proliferation medium supplemented with CM (5%) or sucrose (0-3% and 7-10%); MS medium fortified with GA, IAA or NAA and proliferation medium supplemented with CM (15%) induced excessive callusing at the base of the shoot buds. Proliferation medium supplemented with CH, U and Tri promoted elongation however it also resulted in fragile, yellowing and shedding of leaves thus they were not used for further study. However, proliferation medium supplemented with CM (10%) promoted elongation. In 8 weeks the length of the shoots was 2.76, 2.70, 2.26 and 2.83 cm in *Carica papaya* L. varieties Co-5, Madhur, Pusa Dwarf and Washington respectively (Table 1).

Rooting was not observed in shoots inoculated on MS medium fortified with NAA (1-5.0 mg/l) or base of the shoot dipped in NAA, IAA or IBA (250-3000 mg/l) for 10 and 20 seconds and inoculated on MS medium. Profuse callusing was observed at the base of the shoots, which were dipped in NAA and IAA.

<table>
<thead>
<tr>
<th>Varieties</th>
<th>Co-5</th>
<th>MA</th>
<th>PD</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot bud numbers</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induction media</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAP + AS + NAA (mg/l)</td>
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<td>Proliferation media</td>
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<td>BAP + AS + NAA + Sucrose (6%)</td>
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<td>4.0±10±2.0</td>
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<tr>
<td>0.97±0.14</td>
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<td>5.0±10±2.0</td>
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<tr>
<td>1.61±0.09</td>
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<tr>
<td>1.03±0.12</td>
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<td>Elongation media</td>
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<tr>
<td>BAP + AS + NAA + CM (10%)</td>
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<td></td>
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<tr>
<td>5.0±10±2.0</td>
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<td></td>
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</tr>
<tr>
<td>2.76±0.11</td>
<td>2.70±0.07</td>
<td>2.26±0.32</td>
<td>2.83±0.20</td>
<td></td>
</tr>
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</table>

Values are mean of three sets of determinants. Each set containing 10 explants.

Abbreviations used in table and text: BAP: 6-Benzyleaminopurine; AS: Adenine sulphate; NAA: α-Naphthyleneacetic acid; Co-5: Coimbatore-5 variety; MA: Madhur variety; PD: Pusa Dwarf variety; W: Washington variety; MS: Murashige and Skoog medium (1962); MS + B5, ½ MS media and proliferation medium supplemented with CM (5%) or sucrose (0-3% and 7-10%); MS medium fortified with GA, IAA or NAA and proliferation medium supplemented with CM (15%) induced excessive callusing at the base of the shoot buds. Proliferation medium supplemented with CH, U and Tri promoted elongation however it also resulted in fragile, yellowing and shedding of leaves thus they were not used for further study. However, proliferation medium supplemented with CM (10%) promoted elongation. In 8 weeks the length of the shoots was 2.76, 2.70, 2.26 and 2.83 cm in *Carica papaya* L. varieties Co-5, Madhur, Pusa Dwarf and Washington respectively (Table 1).
(250-3000 mg/l) for 30 seconds and inoculated on MS medium. The shoots inoculated on MS medium fortified with IAA or IBA (1.0-5.0 mg/l) produced roots however % rooting was low and time taken for rooting was more, thus these rooting media were not used for further study. The base of the shoot dipped in IBA (2500 mg/l) for 30 seconds, inoculated on MS basal medium showed 90.3, 89.3, 64.6 and 91.0 % rooting in 15, 16.6, 16 and 14 days in Carica papaya L. varieties Co-5, Madhur, Pusa Dwarf and Washington respectively. In the green house combination of soil: vermiculite: cowdung (1: 1: 1) used as a substratum could show 42, 52, 16 and 50 % survival of plantlets in Carica papaya L. varieties Co-5, Madhur, Pusa Dwarf and Washington respectively in 4 weeks (Table 2).

In the present study the axillary bud explants (Fig. A) of Carica papaya L. varieties Co-5, Madhur, Pusa Dwarf and Washington inoculated on MS medium (6% sucrose) fortified with BAP (5.0 mg/l), AS (10 mg/l) and NAA (2.0 mg/l) showed shoot bud initiation and proliferation. Proliferation media supplemented with CM (10%) promoted the elongation of the multiple shoots (Fig. B). *In vitro* culture of Carica papaya L. cv. ‘Rajshahi-red’ was initiated by culturing shoot apices from mature plant onto MS medium supplemented with 2ip (10 mg/l) + NAA (1.0 mg/l) and multiple lateral bud proliferation was induced after transferring into half the strength of MS medium (4 weeks) having BA (0.1 mg/l) + NAA (0.02 mg/l) (Hossain *et al*., 1991). In the present study root initiation after dip treatment in IBA (2500 mg/l) for 30 seconds inoculated on MS basal medium showed % rooting (Table 2).

<table>
<thead>
<tr>
<th>Varieties</th>
<th>Rooting (%)</th>
<th>Time taken (d)</th>
<th>Substratum - Soil: vermiculite: cow-dung (1:1:1) in green house in 4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-5</td>
<td>90.3 ± 0.57</td>
<td>15 ± 1.00</td>
<td>Hardening success (%) 42 ± 1.87</td>
</tr>
<tr>
<td>MA</td>
<td>89.3 ± 1.52</td>
<td>16 ± 0.57</td>
<td>52 ± 0.57</td>
</tr>
<tr>
<td>PD</td>
<td>64.6 ± 1.52</td>
<td>16 ± 2.64</td>
<td>12 ± 1.15</td>
</tr>
<tr>
<td>W</td>
<td>91.0 ± 1.00</td>
<td>14 ± 1.73</td>
<td>50 ± 1.33</td>
</tr>
</tbody>
</table>

Values are mean of three sets of determinants. Each set containing 10 explants.

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**Table 2**: Rooting and hardening of the multiple shoots in different varieties of Carica papaya L.

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**Fig. A**: Carica papaya L. variety Co-5-axillary bud.

**Fig. B**: Carica papaya L. variety Madhur - multiple shoots.

**Fig. C**: Carica papaya L. variety Pusa dwarf – rooting.

**Fig. D and E**: Carica papaya L. variety Washington–Hardening in green house and sfield.
maximum rooting (Fig. C). These plantlets were transferred to soil: vermiculite: cowdung (1: 1: 1) in the hardening chamber, green house and finally to the field (Fig. D and Fig. E). In dioecious variety (MF-1), IBA dip treatment given to cut end of shoot produced healthy root system within a month. A successful in vitro to in vivo transfer of 100 % plantlets were achieved in substrate mix of Vermiculite: Celrich: Red soil: Peat and Perlite in ratio of 15: 50: 15: 15 (Bankar and Sharon, 1996).

Carica papaya L. is an excellent example of a crop that is extremely vulnerable, but economically very important. Vegetative propagation methods do not exist for large-scale plantations, hence clonal propagation through tissue culture is much desired. Economically important Carica papaya L. cultivars Co-5, Pusa Dwarf and Washington could be successfully regenerated in vitro by somatic embryogenesis (Saha et al., 2004). In the present study an attempt has been made to develop a protocol for the clonal propagation of dioecious varieties of Carica papaya L. through organogenesis by the direct multiple shoot formation from the axillary bud explants of the field grown plants.

REFERENCES

Abstract: The aim of the present study was to evaluate the effect of aflatoxin on histopathological and biochemical changes in the vas deferens of mice and its possible amelioration on pretreatment with vitamin E. Adult male albino mice were orally administered with 25 and 50 μg of aflatoxin/animal/day for 45 days. Vas deferens was isolated and processed for histopathological and biochemical analysis. Results revealed dose-dependent, significant reductions, as compared with controls, in absolute and relative weights of vas deferens. Histopathological studies revealed pyknosis in the nuclei of the epithelium and an increase in the thickness of lamina propria in vas deferens of aflatoxin-treated mice. The stereocilia were clumped and the lumen did not contain the sperms. While glycogen and protein contents were significantly reduced, the phosphorylase activity was higher in aflatoxin-treated mice. Vitamin E (2 mg/animal/day) pretreatment significantly restored the aflatoxin-induced histopathological and biochemical changes. The recovery was more in low dose intoxicated mice than that of high dose. The results showed that vitamin E pretreatment significantly ameliorates aflatoxin-induced changes in vas deferens of mice.

Keywords: Aflatoxin, Vitamin E, Vas deferens.

INTRODUCTION

Aflatoxins (B₁, B₂, G₁, G₂) are secondary toxic fungal metabolites produced by Aspergillus flavus and A. parasiticus. They contaminate various food/feed stuffs. Concentration of aflatoxin in various food/feed stuffs is much higher in tropical and subtropical countries due to congenial environmental conditions for mold growth and aflatoxin production. Aflatoxins are also found in edible tissues, milk and eggs of farm animals after ingestion of aflatoxin contaminated food (Fink, 1999).

Aflatoxin B₁ is activated to AFB₁-8, 9-exoepoxide and forms adduct primarily at N₇ position of guanine and are responsible for its mutagenic and carcinogenic effects (Groopman et al., 1996). In addition, the lipid peroxidation and oxidative DNA damage are also manifestations of aflatoxin B₁ induced toxicity (Shen et al., 1994).

Male fertility has deteriorated in many countries during the last few decades due to poor sperm quality (Irvine et al., 1996; Toppari et al., 1996). Picha et al. (1986) have also reported in experimental animals that semen quality is adversely affected on administration of aflatoxin. Though the vas deferens, which helps in transmission of sperms from its storage site, is an important part of reproductive system, literature reveals no report on the effect of aflatoxin on this tissue.

The aim of the present investigation was to evaluate the effect of aflatoxin administration on histopathological and biochemical changes in vas deferens of mice and its possible amelioration on pretreatment with vitamin E. The later is a potent biological antioxidant and known to inhibit lipid peroxidation in biological membranes by scavenging the chain-propagating peroxyl radicals (Elena et al., 1990).
MATERIALS AND METHODS

Aspergillus parasiticus (NRRL 3240) obtained from the Indian Agricultural Research Institute, New Delhi, India was grown on sucrose-magnesium sulphate-potassium nitrate-yeast extract (SMKY) liquid medium at 28±2 °C for 10 days (Diener and Davis, 1966). Culture filtrates were extracted with chloroform (1:2, v/v) and passed through anhydrous sodium sulphate. The extract was evaporated to dryness.

Dried aflatoxin extract was dissolved in fresh chloroform and used for chemical analysis. 100 µl aflatoxin extract was fractionated on silica gel G coated activated TLC plates along with standard (a gift from International Agency for Research on Cancer, Lyon, France). The plates were developed in a solvent system consisting of toluene: iso-amyl alcohol: methanol (90:32:2, v/v). The air-dried plates were observed under long-wave UV light (360 nm) for aflatoxins. Different components of aflatoxins were initially identified visually by comparing colour and intensity of fluorescence as well as polarity of sample spots with standards. Chemical confirmation of aflatoxin was done by spraying trifluoroacetic acid and 25% sulfuric acid.

Each spot was scraped separately and dissolved in chilled spectrophotometric grade methanol. After centrifugation for 10 min at 1000 g the UV absorption spectrum of methanolic solution was determined using UV-Vis spectrophotometer (Nabney and Nesbitt, 1965). Dried aflatoxin extract containing B1, B2, G1 and G2 in the percentage of 57:22:14:7 respectively was dissolved in olive oil and administered orally by gastric intubation in experimental animals.

Young inbred Swiss strain male albino mice (Mus musculus) weighing approximately 32-34 g were obtained from Cadila Health Care, Ahmedabad, India. Animals were provided with animal feed (prepared as per the formulation given by the National Institute of Occupational Health, Ahmedabad, India and water ad-libitum). The animals were maintained under 12-h light/dark cycles at 25±2°C. Guidelines for care and use of animals in scientific research -1991 published by Indian National Science Academy, New Delhi, India was followed.

Seventy such animals were randomly divided into seven groups and caged separately. Group 1 animals were maintained without any treatment (untreated control). Animals of groups 2 and 3 received olive oil (0.2 ml/animal/day) and vitamin E (2 mg/0.2 ml olive oil/animal/day) respectively for 45 days and served as pretreatment controls. Animals of groups 4 and 5 were orally administered (by gastric intubation) with 25 and 50 µg aflatoxin/0.2 ml olive oil/animal/day (750 and 1500 µg/kg body weight; 1/12th and 1/6th of LD50 value) respectively for 45 days. In addition to aflatoxin treatment as mentioned for groups 4 and 5 animals, animals of groups 6 and 7 also received vitamin E (2 mg/0.2 ml olive oil/animal/day) orally 1 h prior to administration of aflatoxin for 45 days.

Vitamin E was obtained from Hi Media Laboratories Ltd., Mumbai, India. For administration, aflatoxin and vitamin E were dissolved in olive oil. Hence, it was used as a vehicle in group 2 animals. Vitamin E (2 mg/0.2 ml olive oil/animal/day) was administered in group 3 animals to see its effect alone, if any. As different isomers of aflatoxin exist together in the food-stuffs, we preferred to carry out the experiment with mixed aflatoxins. The dose of aflatoxin was based on LD50 value i.e., 9 mg/kg body weight for male mice as recommended by Smith and Moss (1985). The effective dose of vitamin E was based on earlier work by Chinoy and Sharma (1998). Duration of the treatment (45 days) was based on cumulative toxicity of aflatoxin (Markarananda et al., 1998).

On completion of the treatment, mice were sacrificed by cervical dislocation. The vas deferens was quickly isolated, blotted free of blood and weighed to the nearest mg on a balance. Relative weight was calculated. Histopathological studies were carried out by the standard technique of hematoxylin-eosin staining. The samples of vas deferens were fixed for 18 h in alcoholic bouin’s fixative. The tissues were dehydrated by passing through ascending grades of alcohol, cleared in xylene and embedded in paraffin wax. 5 µm thick sections were cut on a rotary microtome and stained with Ehrlich’s hematoxylin-eosin (alcohol soluble), dehydrated in alcohol, cleared in xylene, mounted in DPX and examined microscopically.

The concentration of protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. The glycogen concentration in vas deferens was estimated by the method of Seifert et al. (1950). The activity of phosphorylase (E.C. 2.4.1.1) was assayed by the method of Cori et al. (1943). The inorganic phosphorus liberated was estimated by the method of Fiske and Subbarow (1925).
**Statistical analysis:** Results are expressed as means ± SEM. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s test. The level of significance was accepted with p<0.05. Comparisons of p-values between different groups were performed.

**RESULTS**

No significant changes were observed between the control groups (Group 1, 2, 3). Oral administration of 25 and 50 μg aflatoxin/animal/day for 45 days caused a significant dose-dependent reduction in absolute and relative weight, as well as, protein and glycogen contents in vas deferens of mice as compared to controls. Contrary to these, the phosphorylase activity was significantly higher in aflatoxin-treated animals. Vitamin E pretreatment significantly recovered the aflatoxin-induced alterations, but only in low dose intoxicated mice. In high dose aflatoxin-treated mice the amelioration with vitamin E was partial (Table 1).

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Untreated control 1</th>
<th>Vehicle control 2</th>
<th>Vit. E control 3</th>
<th>Low dose aflatoxin 4</th>
<th>High dose aflatoxin 5</th>
<th>Vit. E + low dose aflatoxin 6</th>
<th>Vit. E + high dose aflatoxin 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameters</strong></td>
<td>Absolute weight (mg)</td>
<td>Relative weight (mg/100 gm body weight)</td>
<td>Protein (mg/100 mg tissue weight)</td>
<td>Glycogen (mg/100 mg tissue weight)</td>
<td>Phosphorylase activity (µg phosphorus released/mg protein/15 min)</td>
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<tr>
<td></td>
<td>24.21 ±0.87</td>
<td>72.44 ±0.91</td>
<td>13.31 ±0.48</td>
<td>1.52 ±0.02</td>
<td>8.04 ±0.18</td>
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<tr>
<td></td>
<td>24.79 ±0.72</td>
<td>71.54 ±0.90</td>
<td>13.36 ±0.53</td>
<td>1.49 ±0.02</td>
<td>8.03 ±0.02</td>
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<tr>
<td></td>
<td>25.00 ±0.68</td>
<td>71.55 ±0.90</td>
<td>12.99 ±0.53</td>
<td>1.47 ±0.02</td>
<td>7.92 ±0.24</td>
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<td></td>
<td>20.62 ±0.85abc</td>
<td>61.13 ±0.90abcf</td>
<td>11.08 ±0.43abc</td>
<td>1.22 ±0.04abcf</td>
<td>14.55 ±0.33abcf</td>
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<td>17.52 ±0.88abcdefg</td>
<td>57.77 ±0.98abcdef</td>
<td>9.81 ±0.54abcf</td>
<td>1.12 ±0.03abcdefg</td>
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<td>24.82 ±0.75abc</td>
<td>72.75 ±0.95abcf</td>
<td>12.82 ±0.58abcf</td>
<td>1.51 ±0.04abcf</td>
<td>9.37 ±0.40</td>
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<td>18.64 ±0.93abcdefg</td>
<td>61.08 ±0.93abcf</td>
<td>11.43 ±0.49abc</td>
<td>1.25 ±0.05abcdefg</td>
<td>13.55 ±0.76abcdefg</td>
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</table>

*a* as compared to the Group 1: p<0.05; *b* as compared to the Group 2: p<0.05; *c* as compared to the Group 3: p<0.05; *d* as compared to the Group 4: p<0.05; *e* as compared to the Group 5: p<0.05; *f* as compared to the Group 6: p<0.05; *g* as compared to the Group 7: p<0.05.

**Table 1:** Effect of pretreatment with vitamin E on aflatoxin-induced changes in the vas deferens of mice (means ± SEM, n= 10)

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**Transverse sections of vas deferens:**

**Fig. 1 (Control):** Note the normal pseudostratified epithelium with stereocilia and prominent epithelial folds. The lumen appeared stellate with presence of sperms. The muscle layer and lamina propria are also seen (X 510).

**Fig. 2 (Aflatoxin treated):** Epithelium showed nuclear pyknosis. The stereocilia were clumped and the lumen contained no sperms. The lamina propria was increased in thickness with foldings as compared to control (X 510).

**Fig. 3 (Aflatoxin + vitamin E treated):** Note the recovery evidenced by the presence of stereocilia and normalcy in the thickness of lamina propria with no folds but no sperm bundles (X 510).
The vas deferens of mice consists of three muscular layers, namely the outer longitudinal, middle circular and inner longitudinal layers. The lamina propria is present between the inner longitudinal muscle layer and the pseudostratified epithelium, which contained stereocilia on the luminal surface. The epithelial cell layers are folded so as to form a stellate lumen in which the sperm bundles are present (Fig. 1).

Oral administration of aflatoxin for 45 days caused nuclear pyknosis and degeneration in epithelium. The lamina propria increases in thickness. The stereocilia are clumped and the lumen did not contain the sperms (Fig. 2).

The vas deferens of mice given pretreatment with vitamin E along with aflatoxin significantly neutralized the aflatoxin effects. In such animals a normal epithelium and lamina propria were observed. Normal stereocilia were also seen but sperm bundles were missing in the lumen (Fig. 3).

**DISCUSSION**

Aflatoxin intoxication causes a significant reduction in absolute and relative weight of vas deferens which could be due to i) degenerative changes in the wall of vas deferens, ii) absence of sperm bundles in the lumen and iii) biochemical alterations in the tissue. Degenerative changes and pyknotic nuclei in the vas deferens revealed in histopathological studies could be due to oxidative damage and lipid peroxidation which are manifestations of aflatoxin-induced toxicity. Such changes have been reported by earlier investigators (Kaden et al., 1987; Verma and Raval, 1991).

Vitamin E pre-treatment significantly ameliorated aflatoxin–induced alterations in the liver, skeletal muscle and blood (Verma and Nair, 1999). The protective effect of vitamin E on lipid peroxidation in the liver and kidney of aflatoxin-treated mice has been reported by us (Verma and Nair, 1999). The antioxidative function of vitamin E is mainly due to its reaction with membrane phospholipid bilayers to break the chain reaction initiated by hydroxyl radical (Shen et al., 1994). Thus vitamin E appears to reduce aflatoxin-toxicity by reducing oxidative damage.

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