SEX-ASSOCIATED DIFFERENCES IN PEROXIDASES AND ETHYLENE PRODUCTION AND THEIR MODIFICATION BY ETHEPHON TREATMENT IN THE FLOWERS OF *CANNABIS SATIVA* L.

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ABSTRACT

A study of qualitative and quantitative changes in peroxidases (EC 1.11.1.7) in the male and female flowers of dioecious *Cannabis sativa* (each at three developmental stages), showed that male flowers were characterized by the presence of three specific iso-peroxidases and that peroxidase activity was higher in female flowers. More ethylene was evolved by female flowers than by male flowers, especially at the first two stages. Treatment of male plants with ethephon was able to elevate the levels of peroxidases and ethylene production in the induced female flowers. However, the three iso-peroxidases associated with the control male flowers were absent in the female flowers induced on male plants but were present in the intersexual flowers.

INTRODUCTION

LIKE all morphogenetic processes, the development of flowers of different sexes, either on the same or on different individuals, is under the influence of hormones[1]. Whereas the relative endogenous balance of ethylene and gibberellins has been suggested to control sex expression in *Cannabis sativa*[2], interactive involvement of other hormones such as auxins and cytokinins in *Mercurialis annua*[3], gibberellins and cytokinins in *Spinacea oleracea* and *C. sativa*[4] in the differentiation of sex organs have also been reported. In *C. sativa*, female flowers can be induced on male plants by exogenous application of ethephon, an ethylene releasing compound[5].

Specific iso-peroxidases have been associated with male sex expression in *M. annua*[6] and *Coccinia indica*[7]. Certain tyrosyl- and leucyl-*t*RNA species[8], one immunologically detectable antigen[9] and a few iso-esterases[6] have been noted exclusively in the female flowers of *M. annua*. Such differences are interpreted as manifestations of differential gene activity, although their regulation and precise role in sex differentiation have not been understood. Hormones could be playing a regulatory role in gene activity and in the synthesis of macromolecules.

In this paper, we have compared the qualitative and quantitative changes in peroxidases (EC 1.11.1.7) in the male and female flowers of *C. sativa* as also in the ethephon-induced intersexual and female flowers on male plants to determine whether peroxidases are associated with the flower sex type and if so, whether sex reversal induced by ethephon would cause a correlative change in the peroxidases.

MATERIALS AND METHODS

Two to three-week old seedlings of *Cannabis sativa* (*ca* 10–15 cm in height) growing naturally in the Botanical Garden of the Department of Botany, University of Delhi were transplanted individually to earthen pots and grown under field conditions. These formed the source of the samples detailed below.

Treatment. A set of 20 male plants were sprayed with an aqueous solution of ethephon (ethrel, CEPA or 2-chloroethylphosphonic acid; Agromore Ltd., Bangalore, India) at a concentration of 1920 ppm for inducing female flowers[5]. Intersexual and female flowers started appearing after 10–15 days of treatment.

Samples. The plant materials used for analysis were: male and female flowers each at three stages of development (MB1, MB2 and MB3 for male and FB1, FB2 and FB3 for female; see table 1), 2 cm long shoot tips from the male and female plants (comprising the shoot apex, six to eight condensed nodes with young leaves and male or female flowers), pollen grains, mature seeds and intersexual flowers and induced female flowers at stage FB2 on ethephon-treated male plants.

Protein extraction. 200 mg of the fresh material was extracted in 2.0 ml of ice-cold 50 mM K-phosphate

buffer (pH 7.4) containing 5 mM EDTA and 0.02% β-mercaptoethanol. After centrifugation (0°C) for 45 min at 20,000 g, the supernatant was used as the crude protein extract.

Protein and enzyme assays. The total protein content in the crude extract was determined following the method of Bradford[10]. Peroxidase activity was estimated using hydrogen peroxide as the substrate and guaiacol as the hydrogen donor[11]. One unit of peroxidase has been defined as that amount which causes a δ absorbance of 0.1 (at 420 nm) in 1 min at 26°C.

Crude extracts containing 200 μg of protein were subjected to discontinuous gel electrophoresis[12] using 7.5% polyacrylamide gels, at a constant current of 4 mA per tube (10.5 cm long and 0.5 cm dia) for 1.5 hr. The gels were incubated in a mixture containing 0.6 ml of guaiacol and 0.5 ml of 3% hydrogen peroxide in 100 ml of 0.2 M Na-acetate buffer (pH 4.0) for visualizing the isoenzymes of peroxidase.

Ethylene estimation. Ethylene evolution was estimated only from flowers. Fifteen flowers (for each stage) were excised, weighed and transferred immediately to small gas chambers (designed in our laboratory) containing 0.05 ml of distilled water. The total volume inside the gas chambers was 2.5 ml. After incubation for 6 hr at 26°C, ethylene in the gas samples was estimated using an Aimil-Nucon 5500 digital gas liquid chromatograph, Nucon Engineers, India.

RESULTS AND DISCUSSION

At all stages of development studied, female flowers showed higher total and specific activities of peroxidases than the male flowers (table 2). The peroxidase activity declined steadily with age in the male but not in the female flowers.

In cucumber, gynocecious plants show greater activities of peroxidases and IAA-oxidases than the monoecious plants[13]. Jaiswal and Kumar[7] have shown that the quantity of peroxidases in the female flower primordia of *Coccinia indica* is higher than in the male primordia. These and other authors[14–16], have argued that peroxidases play an enzymic role in the biosynthesis of ethylene from methionine and its derivatives. That female flowers of *C. sativa* show greater peroxidase activity than male flowers, and that they also evolve more ethylene on a unit fresh weight basis (table 2) support the above argument. However, there is recent evidence[17–20], that ethylene is generally produced from methionine *via* methionine → → → SAM (S-adenosyl methionine) → → → ACC (1-aminocyclopropane carboxylic acid) pathway with no specific role for peroxidases. Hence further detailed investigations are required before a definite relationship can be established between peroxidases and ethylene. This assumes even greater significance from our observations as well as those of Ridge and Osborn[21], Imaseki[22] and Retig and Rudich[13] that treatment with ethephon increases the activity of peroxidases. In the present work this is evidenced in the ethephon-induced female flowers on male plants that show greater activity of peroxidases compared with the control male flowers (table 2). Curiously the ethephon-induced intersexual flowers resembled normal male flowers in peroxidase activity.

The observation that natural female flowers as well as ethephon-induced female flowers of *C. sativa* evolved significantly higher amounts of ethylene than the male flowers at younger stages, also strengthens the concept that a higher amount of ethylene in the female plants is responsible for female sex expression in this plant. Such a concept has been proposed earlier on the basis of exogeneous application of ethylene-releasing com-

Table 1 *Details of developmental stages of flowers/fruits sampled.*

Stage	Average size (length in mm x breadth in mm)	Average fresh weight (mg)	Characteristic features
MB 1	1.0 × 1.0	0.46	Male buds with sporogenous cells in the anthers
MB 2	2.0 × 1.5	1.45	Buds undergoing microsporogenesis
MB 3	3.5 × 2.5	6.60	Buds one day before anthesis containing mature pollen grains
FB 1	6.0* × 1.0	0.58	Unpollinated female flowers with white protruding stigmas
FB 2	6.5* × 2.0	1.60	Flowers after 4–6 days of pollination. Stigmas brown and shrivelled
FB 3	8.0 × 4.0	7.50	Young fruits with liquid endosperm and embryo at globular stage

*The length is from the base of the ovary to the tip of the protruding stigmas

Table 2 *Activity of peroxidases* and ethylene production in the male and female reproductive tissues of Cannabis sativa.*

Material	Total activity of peroxidases (units/g fresh weight) Mean ± S.E.	Specific activity of peroxidase (units/mg protein) protein) Mean ± S.E.	Ethylene evolution (ml/g fresh weight/h) Mean ± S.E.
Male flowers			
MB 1	100.94 ± 5.77	5.15 ± 0.29	11.73 ± 1.56
MB 2	58.80 ± 1.25	4.17 ± 0.09	6.88 ± 1.84
MB 3	25.03 ± 2.89	2.43 ± 0.28	3.53 ± 0.81
Pollen grains	49.95 ± 5.77	2.22 ± 0.26	**
Female flowers			
FB 1	128.03 ± 7.47	18.69 ± 1.09	46.67 ± 2.47
FB 2	260.04 ± 8.54	31.52 ± 2.34	25.21 ± 1.77
FB 3	249.96 ± 8.56	27.17 ± 1.04	5.12 ± 0.65
Seeds	3.98 ± 0.75	0.36 ± 0.07	**
Male shoot tip	169.91 ± 5.00	7.05 ± 0.21	**
Female shoot tip	195.08 ± 6.45	8.99 ± 0.30	**
Intersexual flowers	63.96 ± 2.50	6.21 ± 0.24	16.13 ± 2.09
Induced female flowers	119.99 ± 6.29	10.81 ± 0.57	34.38 ± 2.50

*All values are average of five replicates; **Not estimated

pounds[5,23] and the use of antagonists of ethylene synthesis/action[24,25]. Although ethylene evolution in very young female buds could not be studied (as they could not be isolated without injury), it appears from the present data and those of Jaiswal[23] that the evolution of high amounts of ethylene in early stages of differentiation is important for female sex expression.

Irrespective of the stage of development, male flowers and male shoot tips had three new isoenzymes of peroxidases (figure 1A, B, D, bands *c*, *f* and *g*) over those of the female counterparts. Of these, one band (c) was relatively slow-moving with an R_f range of 0.37 to 0.38 while the other two bands were fast-moving with R_f values ranging from 0.62 to 0.63 and 0.67 to 0.69, respectively.

Significantly, the iso-peroxidases in the ethephon-induced female flowers (figure 1C) showed a pattern typical of normal female flowers. There were no traces of the three isoenzymes associated with the male flowers although these flowers were only phenotypically female and were borne on male plants. The zymogram of ethephon-induced intersexual flowers (figure 1C) resembled that of normal male flowers. In addition, a light-staining extra band with an R_f value of 0.51, between bands *e* and *g*, not present in any other organ was noted. The significance of this band is not clear.

Anodic peroxidases, specific to male flowers (in angiosperms) and male cones (in gymnosperms), have been suggested to play an important role in stamen morphogenesis[26]. Histoimmunologic studies have shown that these iso-peroxidases are characteristic of sporogenous and tapetal tissues and their synthesis occurs early in staminogenesis[27]. Our findings show that the iso-peroxidases associated with the male flowers are more pronounced in the young male flowers and are absent in the pollen grains. Moreover, in the ethephon-induced intersexual flowers of *Cannabis* even the presence of one stamen is able to cause the appearance of these iso-peroxidases.

However, whether the three iso-peroxidases found associated with the male flowers play any role in the male sex expression of *Cannabis* cannot be unequivocally established in the present work, although it appears that ethephon treatment, shuts off the synthesis of these iso-peroxidases in the induced female flowers on male plants. Also, whether ethylene has any direct role in this process has to be investigated.

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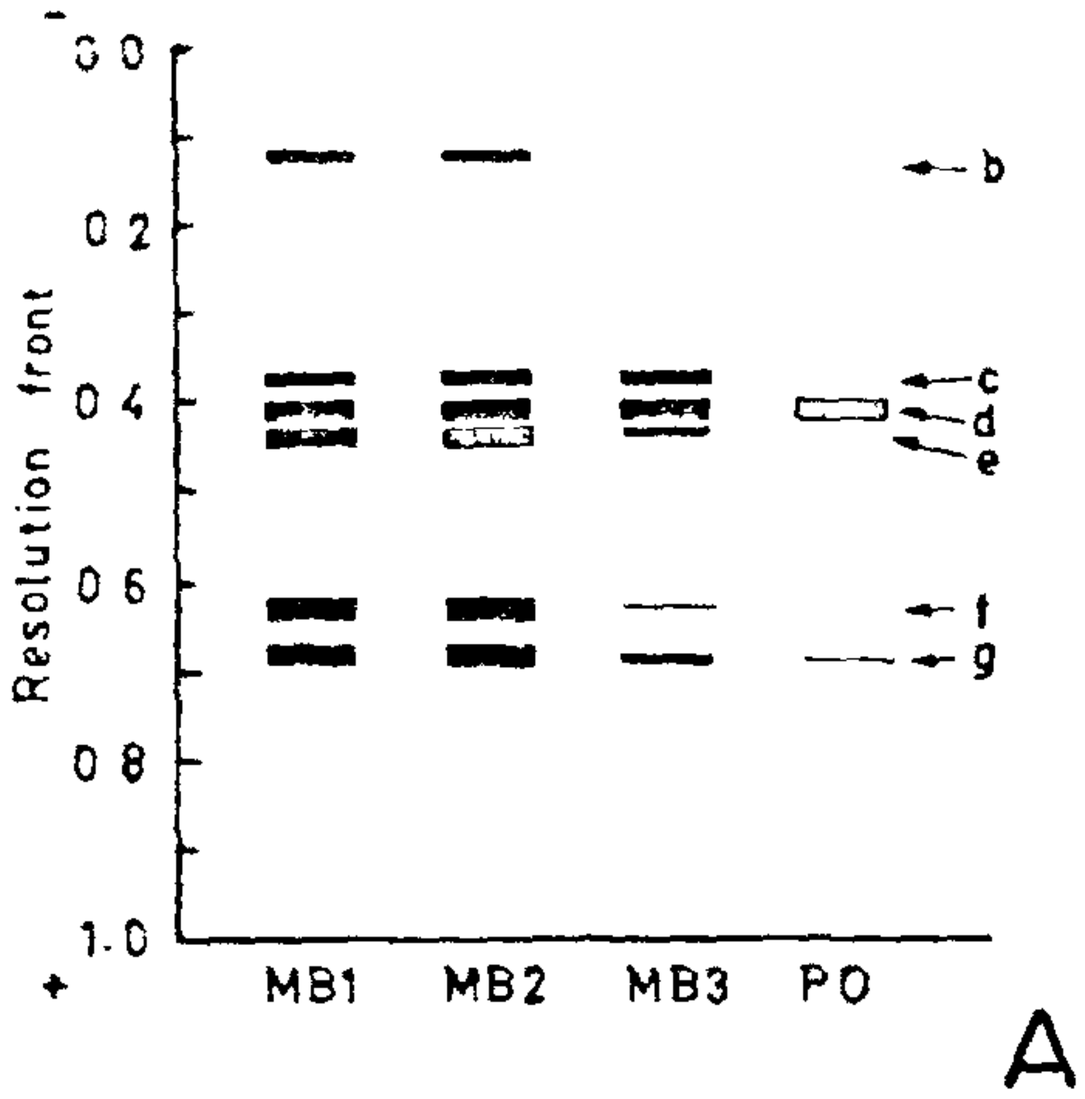

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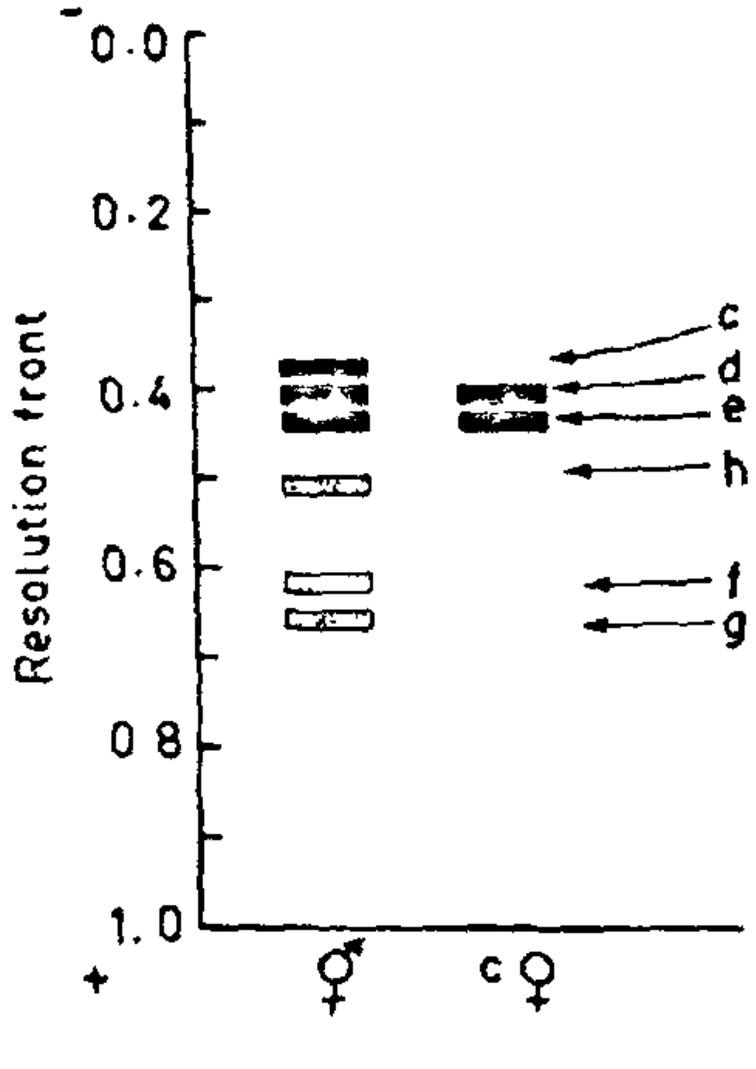

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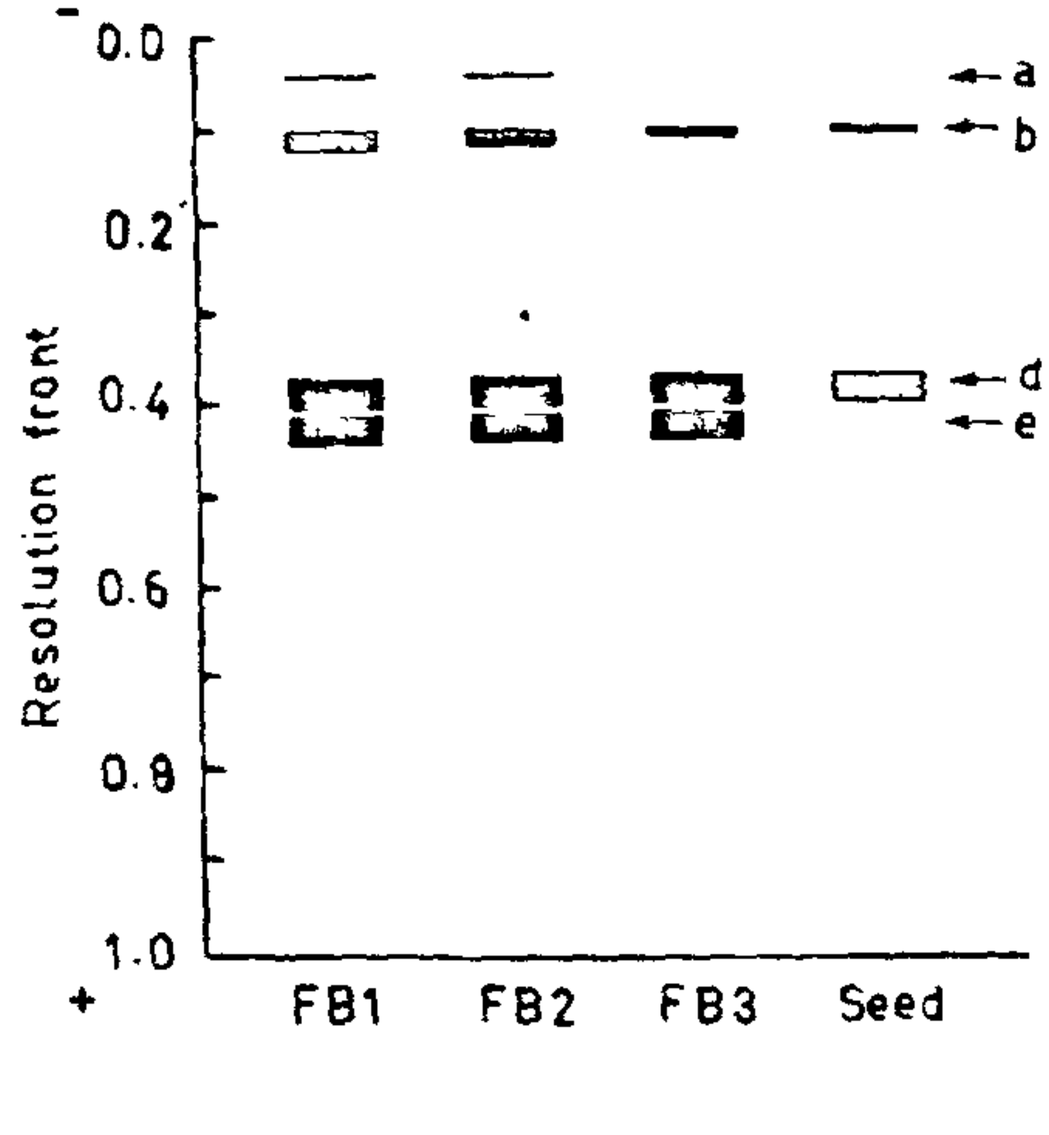

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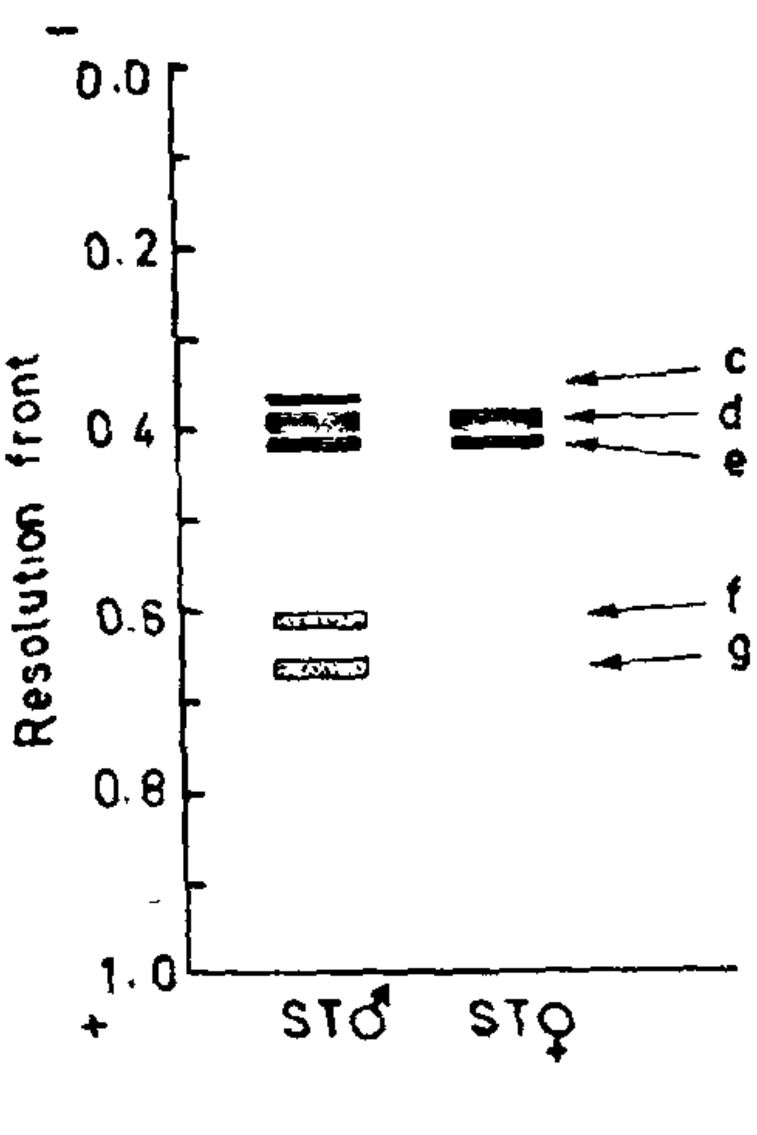

D

Figure 1. Isoenzymes of peroxidase in the reproductive parts of male and female plants of *Cannabis sativa* **A.** In three developmental stages of male flowers (MB1, MB2, MB3) and pollen grains (PO). **B.** In three developmental stages of female flowers (FB1, FB2 and FB3) and seeds. **C.** In the intersexual flowers (♀) and induced female flowers (c♀) on ethephon-treated male plants. **D.** In the male (ST ♂) and female (ST ♀) shoot tips.

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ANNOUNCEMENT

BILATERAL AGREEMENTS: PROPOSALS INVITED FOR 1985

Proposals are invited for international collaboration activities to commence in 1985 under Australia's science and technology agreements with Japan, USA, Mexico, West Germany, India and China. Funding – normally limited to economy return airfares and support for living costs – is available for 'seminars and workshops which have been developed from the outset as proposed agreement activities or short-term visits (up to 6 months) for cooperative projects. It is anticipated that the primary funds for collaboration will be provided by the cooperating agencies themselves. Support is not available for attendance at general international seminars or conferences'.

Priority is given to activities in: bio-technology; biomedical technology; communications technology; information technology; advanced materials technology; manufacturing technology; and marine science. Projects in other areas will be considered.

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