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FORMATION OF PLANTLETS IN CULTURED ANTHERS OF *NICOTIANA TABACUM* CV. VIRGINICA

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Abstract

Anthers of *Nicotiana tabacum* cv. Virginica were cultured and the development of pollen grains studied. A large proportion of pollen grains degenerated in culture, the surviving ones belonging to mitotic and binucleate class produced plantlets which originated from 'S' grains. Cold pretreatment of anthers increased both the number of anthers producing plantlets and the number of plantlets per anther. Dividing the culture period into an initiation and a developmental stage proved beneficial. Addition of mannitol and glutamine gave maximum number of plantlets, absence of which reduced yield. Role of mannitol and glutamine in the development of plantlets from pollen grains in anther culture is discussed.

Introduction

Since the discovery of the formation of haploid plants from pollen grains (Guha & Maheshwari, 1964, Bourgin & Nitsch, 1967) anther culture has gained considerable importance in plant breeding and fundamental research. In studies on the formation of haploids in *Nicotiana* utilizing its different species and cultivars (Sunderland & Wicks, 1971; Sunderland, 1978; Horner & Street, 1978; Misoo & Watanabe, 1985), low yield of plantlets was obtained against an initial population of 40,000 grains per anther. The work on somatic embryogenesis in carrot (Halperin, 1970), *Medicago sativa* (Walker & Sato, 1981) and the formation of apogamous buds on the gametophytes of *Pteridium aquilinum* (Elmore & Whittier, 1975; Khatoon., 1985a) suggested that pollen embryogenesis in *Nicotiana* may also involve more than one step as observed in isolated pollen culture of dihaploid *Solanum tuberosum* (Sopory, 1977). In the present study, the effect of the addition of mannitol and glutamine during these stages on the yield of planlets of *Nicotiana tabacum* cv. Virginica was examined. The whole culture period from anther inoculation to plantlet development was divided into an 'initiation stage' during which pollen grains begin to divide and a 'development stage' during which dividing grains develop into embryoids and then into plantlets.

Material and Methods

Cultivation of donor plants: Seeds of *Nicotiana tabacum* cv. Virginica obtained from Thompson & Morgan, Ipswich Ltd., U. K., were cultivated as described by Khatoon (1985b).

Anther culture: Buds ranging from 11-30 mm in length were surface sterilized for 10 min. in sodium hypochlorite solution (4% available chlorine) and washed with sterile water. Anthers removed aseptically from the buds were floated on culture medium. One anther from each bud was used for the determination of the stage of pollen development at excision. Liquid medium of Murashige & Skoog (1962) without growth hormones containing sucrose @ 40 g/l, pH 5.0, autoclaved at 15 p.s.i. for 15 min was used as basal medium. Five ml culture medium poured into 5 cm diam., plastic Petri dishes was inoculated with 4 anthers.

For cold pretreatment, excised buds were placed in polyethylene bags, covered with aluminium foil and stored at 5 and 10°C. After 3, 7 and 14 days the buds were surface sterilized, anthers aseptically removed and floated on the surface of liquid medium. Mannitol and glutamine were used in the fresh medium. After one week of culture on initiation medium the anthers were removed, blotted free of adhereing medium and transferred to fresh developmental medium for 4 weeks. Inoculated dishes were sealed with Parafilm and stored in transparent boxes lined with thick folds of wet filter papers to maintain high humidity and avoid drying of cultures. Cultures were kept at 25°C in dark for the first 2 weeks then transferred to a 16h photoperiod at a light intensity of 20 W/m^2 provided with white fluorescent tubes for the remaining culture period. Cultures were examined under stereomicroscope and the total number of plantlets arising from each anther counted 5 weeks after the initiation of cultures. Number of light staining 'S' grains and embryoids and the viability of pollen grains was determined as described by Khatoon (1985b).

Results

Staging of anthers: Flower buds of *N. tabacum* cv. Virginica containing anthers at different stages of pollen development were classified into tetrad, uninucleate, mitotic and binucleate classes (Fig. 1). Binucleate class of anthers contained a dimorphic population of pollen grains as found in other cultivars of *N. tabacum* by Sunderland & Wicks, (1971) and Horner & Street (1978). The "S" grains were distinguished by their small size, less cytoplasmic contents, light staining characteristics and absence of starch.

Anther culture: The embryo forming potential of anthers depended on the stage at which they were initially cultured. Anthers containing immature microspores produced embryoids but no plantlets were formed. Mitotic anthers were more productive than the uninucleate and late binucleate anthers (Fig. 2). Early binucleate stage anthers were the most responsive class of anthers as greater number of these anthers produced embryoids and plantlets. As the binucleate grains progressed towards starch deposition the number of anthers producing embryoids or plantlets decreased (Fig. 3).

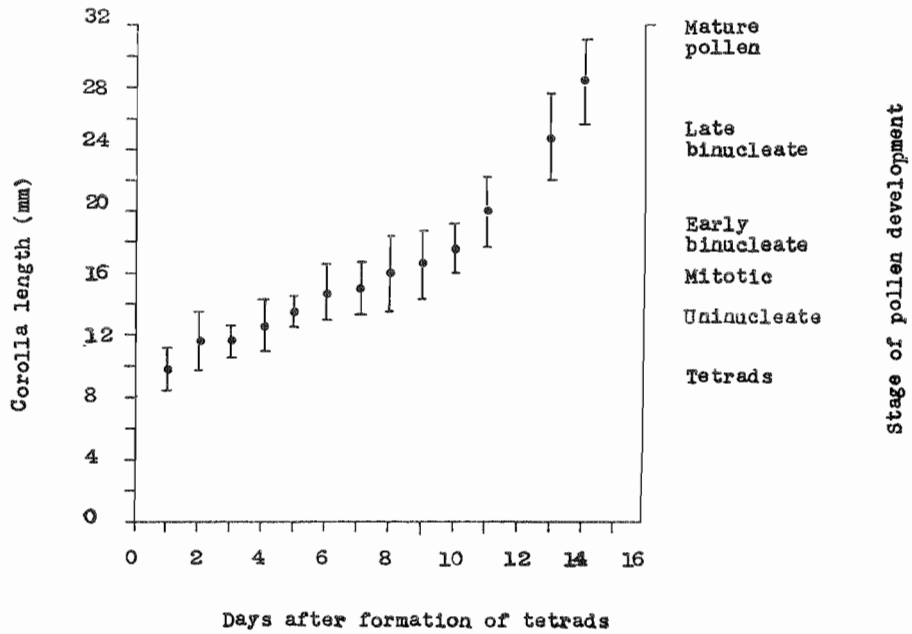

Fig. 1. Relationship between petal length of flower buds and stage of pollen development in anthers of *Nicotiana tabacum* cv. Virginica plants raised in greenhouse.

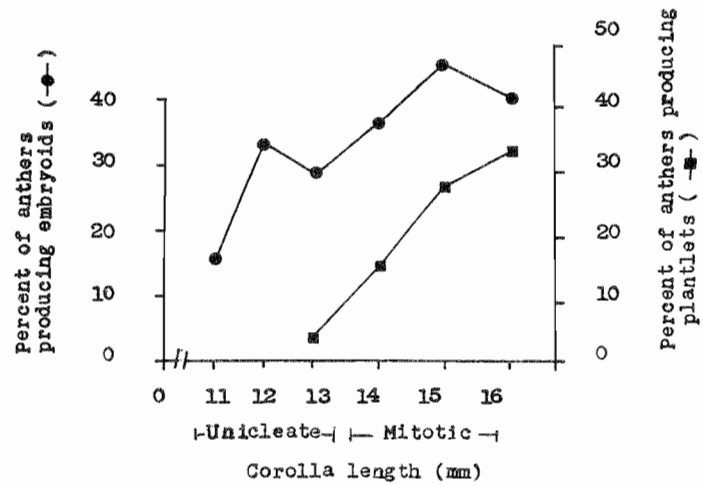

Fig. 2. Frequency of anthers producing embryoids and plantlets in cultured anthers of *Nicotiana tabacum* cv. Virginica at uninucleate (corolla length 11-13 mm) and mitotic stage (corolla length 14-17 mm) of pollen development.

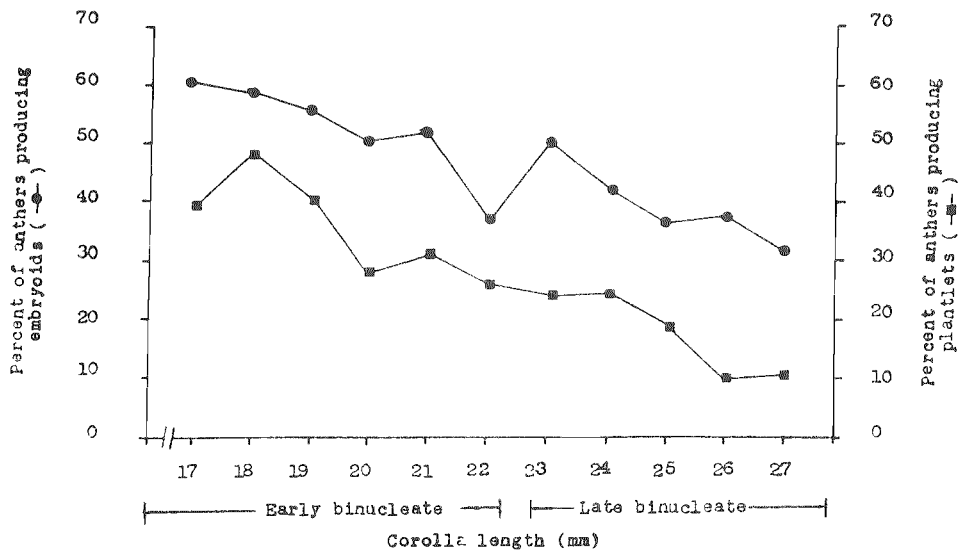

Fig. 3. Fraquency of anthers producing embryoids and plantlets in anther culture of *Nicotiana tabacum* cv. Virginica at late binucleate and early binucleate state of pollen development.

Development of pollen grains in culture: Microspores freshly released from tetrads, obtained from young buds with petal length of 11 mm were generally spherical with moderately thick walls. After 3 days of culture they increased in size, developed thick walls and a big vacuole appeared in their cytoplasm. Anthers obtained from older buds with petal length of 14 mm contained vacuolated microspores. In culture both type of vacuolated microspores went through mitosis, first division being typically asymmetric resulting in the formation of two unequal cells. Some grains remained small in diameter (24-26 μm) stained light and were identified as "S" grains (Table 1).

Mitotic anthers initially contained a mixed population of uninucleate and early binucleate grains. In 3 day old cultures a large number of mitotic grains degenerated and the surviving ones after mitosis became binucleate. In 8 day old cultures, usually 3-celled and occasionally 4-5-celled embryoids were observed. Their number increased between first and second week and approximately 1% population of the grains developed into multicellular embryoids in 3 weeks which passed through heart, torpedo and cotyledonary stages of embryo development. Plantlets emerged after another two weeks with an average of 20 plantlets per anther (Table 1). Leaves of young plantlets expand and turn green, hypocotyls elongate and root hairs develop on roots in culture.

The development of binucleate grains in culture was followed in anthers coming from buds having a petal length between 18-30 mm. 'S' grains were produced in uninucleate, mitotic and early binucleate anthers in culture whereas late binucleate anthers

Table 1. Production of embryoidal grains, embryoids and plantlets in anthers of *Nicotiana tabacum* cv. Virginica at various stages of development.

Stage of pollen development	Petal length (mm)	Days of culture	Viability (%)	'S' grains (%)	Embryoids (%)	Plantlets per anther
Uninucleate	12	0	100	0	0	–
		3	98	0	0	–
		8	57	0	0	–
		14	38	0.3	0.1	–
		21	13	0.5	0.1	–
	14	0	97	0	0	–
		3	89	0	0	–
		8	66	0.1	0	–
		14	31	1.2	0.5	–
		21	11	0.9	1.3	1
Mitotic	15	0	98	0	0	–
		3	56	0	0	–
		8	31	0.1	0.2	–
		14	19	0.2	1.2	–
		21	6	1.2	2.1	20
Early binucleate	18	0	93	0	0	–
		3	87	1	0.05	–
		8	61	1	0.5	–
		14	46	1.7	0.9	–
		21	18	1.0	2.3	11
Late binucleate	30	0	95	1.2	0	–
		3	90	1	0.2	–
		8	83	0.9	–	–
		14	51	0.1	1.1	–
		21	20	0	0.8	8

produced them *in vivo*. The number of 'S' grains in cultured late binucleate anthers never exceeded the one initially present, however, in early binucleate anthers a gradual rise in the number of 'S' grains was observed at the beginning of culture period which gradually declined and disappeared. Several embryoids at multicellular stage were ob-

served which later developed into plantlets with an average of II plantlets per anther (Table 1). Embryoids appear earlier in cultures initiated from the late binucleate anthers. Occasionally vegetative nucleus showing mitotic division with 3-celled embryo could be seen 3 days after the initiation of culture.

Origin of embryoids: The origin of embryoids from 'S' grains was evident. Vegetative nucleus showing mitosis was seen within 3 days after the beginning of culture in late binucleate anthers. The time required for the emergence of 3-celled embryoids varied from 1-2 weeks in mitotic to early uninucleate anthers. Young embryoids remained covered with exine until 32-celled stage when exine burst releasing the embryoids which showed further development. All normal stages of embryo development were observed in cultures.

Pollen degeneration: The rate of loss of viability in pollen grains from both type of binucleate anthers in culture was slow as compared to the pollen grains from uninucleate and mitotic class of anthers. Almost half of the grains remained viable after 2 weeks of culture. Mitotic grains were sensitive to culture conditions showing 50% loss in viability within 3 days (Table 1).

Effect of cold pretreatment: A pretreatment of 5°C for all the three stages increased the frequency of anthers producing plantlets and the mean number of plantlets per anther over the control (Table 2). Highest yield of plantlets per anther was obtained from mitotic anthers whereas high percentage of productive anthers was found in binucleate class after 2 weeks pretreatment which was less effective when late uninucleate class of anthers were used. A pretreatment of 10°C for 7 days was more effective for all classes of anthers. Prolonging the duration of pretreatment reduced both the frequency of anthers producing plantlets and the number of plantlets per anther. Maximum anther response was noticed with the early binucleate class of anthers pretreated for 7 days whereas maximum productivity was observed with the mitotic class of anthers.

Effect of mannitol and glutamine: Media supplemented with mannitol and glutamine during the initiation and development stages proved beneficial for the culture of anthers pretreated at 5°C and 10°C (Table 3). Maximum number of plantlets developed from both mitotic and binucleate anthers where the medium was supplemented with 0.5 M mannitol during initiation stage and 5 g/l glutamine during development stage. When glutamine was withdrawn from the medium during development stage of culture plantlet yield was reduced to almost 1/2 in mitotic anthers and 1/3 in the case of binucleate anthers. Omission of mannitol from the initiation stage significantly decreased yield. The binucleate anthers produced almost double the number of plantlets than the mitotic anthers provided glutamine was present during the development stage. Anthers pretreated at 10°C for 7 days gave better yield than the anthers pretreated at the same stage of development at 5°C for 14 days.

Table 2. Effect of cold pretreatment on anther response and anther productivity of *Nicotiana tabacum* cv. Virginica anthers cultured at various stages of development.

Stage of pollen development at excision	Cold pretreatment		No. Initially cultured	Plantlet formation	
	Days	Temp. (°C)		% anthers	Mean No./anther
Late Uninucleate	0	–	80	5.0	1.0
(14 mm)	3	5	80	6.3	1.0
		10	80	–	–
	7	5	80	11.3	3.1
		10	80	12.5	3.8
	14	5	80	15.0	5.3
		10	80	8.8	3.1
Mitotic	0	–	80	28.8	25.6
	3	5	80	–	–
		10	80	36.3	32.7
	7	5	80	38.8	35.3
		10	80	42.5	50.9
	14	5	80	47.5	38.0
		10	80	31.1	28.1
Early binucleate	0	–	80	47.5	12.3
(18 mm)	3	5	80	61.3	17.1
		10	80	68.8	21.7
	7	5	80	62.5	26.3
		10	80	71.3	37.6
	14	5	80	82.5	36.0
		10	80	57.5	14.1

Discussion

Productive anthers in Virginica cultivar were encountered during and just after first mitosis. Early binucleate anthers produced dimorphic population of pollen grains as reported for cv. White Burley (Sunderland & Wicks, 1971) and cv. Wisconsin 38 (Horner & Street, 1978). Embryogenic grains were seen as early as 3 days after culture. Cold pretreatment increased the incidence of pollen embryogenesis. Buds pretreated at 5 and 10°C increased both the number of anthers producing plantlets and the yield of plantlets per anther (Table 2). Prolonged pretreatment at 10°C for 14 days reduced yield. This may be ascribed due to death either during pretreatment or culture and subsequent degeneration of pollen grains.

Table 3. Productivity of pretreated anthers of *Nicotiana tabacum* cv. Virginica in media supplemented with mannitol and glutamine during different stages in culture.

Stage of pollen development	Pretreatment of anthers	Initiation stage* 0.5M mannitol	Development stage** 5 g/l glutamine	Plantlets produced per anther
Mitotic	5°C for	–	–	36.8
	14 days	+	–	68.6
		+	+	116.9
		–	+	37.3
	10°C for	–	–	41.6
	7 days	+	–	82.7
		+	+	132.3
		–	+	55.8
Binucleate	5°C for	–	–	31.4
	14 days	+	–	54.3
		+	+	136.8
		–	+	73.1
	10°C for	–	–	33.4
	7 days	+	–	58.6
		+	+	166.2
			+	88.7

+ = Present; – = Absent; * = Duration one week; ** = Duration four weeks.

Exposure of anthers to various sequences of mannitol and glutamine during initiation and development stages proved beneficial and is suggestive of the involvement of two stages in pollen embryogenesis differeing in their nutritional requirements. The possible role of mannitol appears to create conditions conducive to cell division. It is an inert substance which does not promote pollen germination (Johri & Vasil, 1961) and is known to create condition of water-stress (Imamura & Harada, 1980) which induces ethylene production in intact and excised plant parts (Wright, 1980). Ethylene is a growth hormone and is reported to induce additional cell divisions in maturing pollen grains of wheat (Bennett & Hughes, 1972) and gametophytes of *Pteridium aquilinum* (Elmore & Whittier, 1975), leading to apogamous bud formation. Excised anthers of cv. Burley have also been shown to produce appreciable amounts of ethylene in culture. Binucleate anthers produce more ethylene than the mitotic ones (Horner *et al*, 1977). Although anthers of Virginica cultivar were not analysed for the endogenous ethylene

but the production of large number of plantlets from mitotic and binucleate anthers exposed to mannitol and a considerably high yield from binucleate anthers even in the absence of mannitol (Table 3) is indicative of the similar situation prevailing in cv. Virginica anthers. Mannitol may also act as an osmotic agent as in cell and protoplast culture. Immature zygotic embryos of *Hordeum* (Kent & Brink, 1947) also required a high osmoticum for their survival in culture. A parallel situation may exist with the embryogenic grains too in cultured anthers.

Role of glutamine in providing nutrition to the young developing embryos has been suggested by Nitsch (1974), Sunderland & Roberts (1977) and Sunderland (1979). In the present study highest yield of plantlets was encountered in media containing glutamine provided anthers were treated with mannitol during initiation stage (Table 3). Binucleate anthers produced more plantlets than the mitotic ones. Absence of glutamine reduced yield from both mitotic and binucleate anthers but the number of plantlets produced by mitotic anthers was higher than the binucleate anthers. This differing response of mitotic and binucleate anthers under the two treatments can be explained on the basis of chemical analyses of amino acid content of *Nicotiana* anthers (Weatherhead, 1978; Horner & Pratt, 1979) which suggests that freshly excised or cultured mitotic anthers of *Nicotiana* contain considerably large quantity of endogenous glutamine than the binucleate anthers. This implies that even in the absence of externally supplied glutamine mitotic anthers bear the capability of supporting growth of a considerably large number of embryogenic grains or embryoids or plantlets whereas binucleate anthers may lead to a limited plantlet formation irrespective of the size of available induced grain pool.

References

Bourgin, J. P. and J. P. Nitsch. 1967. Obtention de *Nicotiana* haploides a partir d etamines cultivees *in vitro*. *Ann. Physiol. Veg.*, 9: 377-82.

Bennett, M. D. and W. G. Hughes. 1972. Additional mitosis in wheat pollen induced by ethrel. *Nature* (Lond.), 240: 566-568.

Elmore, H. W. and D. P. Whittier. 1975. The involvement of ethylene and sucrose in the inductive and developmental phases of apogamous buds formation in *Pteridium* gametophytes. *Can. J. Bot.*, 53: 375-381.

Guha, S. and S. C. Maheshwari. 1964. *In vitro* production of embryos from anthers of *Datura*. *Nature* (Lond.), 204: 497-499.

Halperin, W. 1970. Embryos from somatic plant cells. In: *Control mechanisms in the Expression of Cellular Phenotypes* H.A. padykula ed.), pp. 169-191. Symp. Int. Soc. Cell Bio., Academic Press, N. Y., London.

Horner, M., J. A. McComb and A. J. McComb. 1977. Ethylene production and plantlet formation by *Nicotiana* anthers cultured in the presence and absence of charcoal. *J. Exptl. Bot.*, 28: 1365-1372.

Horner, M., and M. L. Pratt. 1979. Amino acid analysis of *in vivo* and androgenic anthers of *Nicotiana tabacum*. *Protoplasma*, 98: 279-282.

Horner, M., and H. E. Street. 1978. Pollen dimorphism – Origin and significance in pollen plant formation by anther culture. *Ann. Bot.*, 42: 763-771.

Imamura, J. and H. Harada. 1980. Effect of abscisic acid and water stress on the embryo and plantlet formation in anther culture of *Nicotiana tabacum* cv. Samsun. *Z. Pflanzenphysiol.*. 100: 285-289.

Johri, B. M. and I. K. Vasil. 1961. Physiology of pollen. *Bot. Rev.*, 21: 325-381.

Kent, N.F. and R.A. Brink. 1947. Growth *in vitro* of immature *Hordeum* embryos. *Science*. 106: 547-548.

Khatoon, K. 1985a. The influence of hydroxyurea on the induction of apogamy in *Pteridium aquilinum* L. *Pak. J. Sci. Ind. Res.*, 28: 348-351.

Khatoon, K. 1985b. Flower induction in *Nicotiana tabacum* cv. Virginica in sterile culture. *Pak. J. Bot.*, 17: 195-204.

Misoo, S. and N. Watanabe 1985. Studies on the mechanisms of pollen embryogenesis: VI. Anther culture in *Nicotiana rustica* and its ancestral species, *Nicotiana paniculata* and *Nicotiana undulata*. *Sci. Rept. Fac. Agr. Kobe Univ.*, 16: 385-390.

Murashige, T. and K. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.

Nitsch, C. 1974. Pollen culture – a new technique for mass production of haploid and homozygous plants. In: *Haploids in Higher Plants: Advances and Potential*. K. J. Kasha (ed.), pp. 123-135. University of Guelph Press, Canada.

Sopory, S. K. 1977. Development of embryoids in isolated pollen culture of dihaploid *Solanum tuberosum*. *Z. Pflanzenphysiol.*, 84: 453-57.

Sunderland, N. 1978. Strategies in the improvement of yield in anther culture. In: *Proc. Symp. Plant Tissue Culture*, Science Press, Peking. pp. 965-986.

Sunderland, N. 1979. Comparative studies of anther and pollen culture. In: *Plant Cell and Tissue Culture: Principles and Applications*. P.O. Larson, E.F. Paddock, V. Raghavan and W.R. Sharp, (eds). pp. 203-219. Ohio State Univ. Press, Columbus.

Sunderland, N. and F. M. Wicks. 1971. Embryoid formation in pollen grains of *Nicotiana tabacum*. *J. Exp. Bot,*, 22: 213-226.

Sunderland, N. and M. Roberts. 1977. New approach to pollen culture. *Nature* (Lond.), 270: 236-238.

Walker, K. A. and S. J. Sato. 1981. Morphogenesis in callus tissues of *Medicago sativa:* the role of ammonium ion in somatic embryogenesis. *Plant Cell Tissue Organ Culture,* 1: 109-121.

Weatherhead, M. A. 1978. *Tissue culture techniques and haploid induction in potatoes.* Ph.D. Thesis, University of Birmingham.

Wright, S. T. C. 1980. The effect of plant growth regulator treatment on the level of ethylene emanating from excised turgid and wilted wheat leaves. *Planta* (Lond.), 118: 381-388.

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