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Bud and Shoot Formation in Juvenile Tissue Culture of Pinus nigra

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Abstract

Adventitious and axillary buds, shoots and needles were generated using tissue cultures from excised embryos of *Pinus nigra* ARNOLD. Formation of these structures in embryo culture was obtained on agar half-strength MS basal medium supplemented with 2 mg. l^{-1} BAP, 250 mg. l^{-1} myo-inositol and NAA and IBA in various concentrations.

Using shoot-tip explants of 20-day seedlings, generation of buds, shoots and needles was also achieved on an agar medium of half-strength MS salts, but supplemented with 1 mg. l^{-1} BAP and 0.001 mg. l^{-1} NAA.

The origins of new buds in these cultures were adventitious, axillary and native from brachyblasts. Long-shoot development and growth of buds and shoots was achieved by transferring them onto a hormone-free medium, sometimes aided by the addition of 0.3% activated charcoal.

The best tissue-media interactions produced bud and shoot formation in 60—70% of cultures.

In-vitro developed shoots were subjected to the same procedures, and typically produced a successive generation of buds, shoots and needles.

Key words: Pinus nigra, tissue culture, auxin, bud induction, shoot formation.

Zusammenfassung

Aus explantierten Embryonen von *Pinus nigra* ARNOLD konnten in Gewebekultur Adventiv- und Achselknospen, Sprosse und Nadeln erzeugt werden. Die Ausbildung dieser Strukturen wurde auf einem halb-konzentrierten MS-Basal-Medium erreicht, dem 2 mg. l^{-1} BAP, 250 mg. l^{-1} Myo-Inositol und NAA und IBA in verschiedenen Konzentrationen zugefügt worden waren. Bei Verwendung von Sproßspitzenexplantaten 20 Tage alter Keimlinge wurde die Ausbildung von Knospen, Sprossen und Nadeln auch erzielt, wenn dem Agar-Medium außer den MS-Salzen 1 mg. l^{-1} BAP und 0,001 mg. l^{-1} NAA zugegeben wurden. Der Ursprung neuer Knospen in diesen Kulturen waren Adventiv- und Achselknospen, die sich aus Kurztrieben entwickelten. Eine Entwicklung von Langtrieben und das Wachstum von Knospen und Trieben wurden erzielt, wenn diese auf ein hormonfreies Medium transferiert wurden, wobei manchmal eine Zugabe von 0,3 prozentiger Aktivkohle hilfreich war. Die beste Gewebe-Medium-Interaktion erbrachte eine Knospen- und Sproßentwicklung von 60—70%. In Vitro entwickelte Sprosse wurden derselben Prozedur unterzogen und produzierten in der Regel so nach und nach Generationen von Knospen, Sprossen und Nadeln.

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Introduction

Practical applications of tissue culture techniques for the vegetative propagation of woody plants have recently been emphasized in several reviews (BONGA 1977, WINTON 1978, SOMMER and BROWN 1979, BOULAY 1980).

SOMMER and BROWN (1974) were the first to report plantlet regeneration from mature embryos in *Pinus* sp., and adventitious bud induction has subsequently been reported for several pine species (BROWN and SOMMER 1977, DAVID and DAVID 1977, WEBB and STREET 1977, TRANVAN 1979, BORNMAN and JANSSON 1980, KONAR and SINGH 1980). Shoots and plantlets have been most effectively produced in tissue cultures of pines using juvenile material — embryos and young seedlings. It is encouraging, however, that recent papers indicate the possibility of regeneration from mature material (FRANCLET *et al.* 1980, BONGA 1981).

In the breeding process, which is particularly long with forest trees, one often gets only a small number of plants with particularly desirable combinations of characteristics. To capture their advantages for practical purposes, such plants must be multiplied many times. Tissue culture provides this capability. European black pine (*P. nigra* ARNOLD) is an economically important conifer species in southern Europe, and vegetative propagation of its elite specimens and/or hybrids is worthy of attention.

For the past decade one of us (B. K-P.) has been working on various methods of tissue culture for this important species, but with little success. Based on our detailed observations, we suspected that *P. nigra* might be among those tree species that are more difficult to differentiate under *in vitro* conditions. This viewpoint was supported by the first series of tissue culture studies, since buds, shoots, or other organized structures failed to differentiate in most media combinations attempted. However, one combination worked moderately well, and led us to the second and third set of experiments. These allow us to report successful induction of buds, shoots and needle structures using two kinds of juvenile tissue of European black pine for culture.

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indoleacetic acid; IBA, indolebutyric acid; NAA, naphtaleneacetic acid.

Material and Methods

Excised mature embryos and shoot tips (1—2 mm of hypocotyl plus cotyledons plus epicotyl) of 20-day-old seedlings of *P. nigra* ARNOLD, were used in these studies. Most are from a Yugoslav native population, but 2 trees known to hybridize well with *P. sylvestris* were used in the third experiment. After being scrubbed under running water with abrasive cloth, seeds were surface sterilized for 20 min in 3% (w/v) Halamid, a comercial product of Na-p-toluenesulfonchloramine with a drop of Tween 20[R] with continuous shaking, and then rinsed four times in sterile distilled water. They were then imbibed in a minimal volume of sterile, distilled water for 24—36 h at 4° C. A second sterilization of the seeds was then performed either in the same way, or for 6 min in 6% (v/v) H_2O_2. The embryos were then immediately dissected out of the seeds and separated from female-gametophyte and other tissue. The seedlings were germinated in petri dishes on sterile moist filter paper in a laboratory, at room temperature. Isolated embryos and seedling explants were cultured on 15 ml agar medium in 25 × 150 mm tubes.

In the first experiment, using excised embryos, the following nutrient media were screened: (1) a basal media with organic components according to SOMMER *et al.* (1975); (2) to SCHENK and HILDEBRANDT (1972); (3) MS medium (MURASHIGE and SKOOG 1962); (4) MS media modified, according to MOMOT (1977); and (5) to CHENG (1975). Sucrose (3%) and Difco Bacto-agar (0.8%) were added to all media. The pH was adjusted to 5.6 before autoclaving. The growth regulator BAP was added to all of the above media at 2 mg. l^{-1}, and IAA, NAA, IBA and 2,4-D were added in various combinations and concentrations.

Tab. 1. — Different media used with excised embryo. All these media were supplemented with 2 mg. l^{-1} BAP, 3% sucrose and 0.8% agar. 24 embryos per treatment used.

	Basal medium mg. l^{-1}	Auxin	mg. l^{-1}
1	SOMMER *et al.* (1975)	IBA	1
2	— " —	NAA	0.1
3	SOMMER *et al.* with 535 NH_4Cl	IBA	1
4	— " —	NAA	0.1
5	MS (MOMOT 1977)	IAA	1
6	— " —	IAA	2
7	— " —	2,4-D	0.5
8	SCHENK and HILDEBRANDT (1972)	IBA	1.5
9	— " —	NAA	1
10	SCHENK and HILDEBRANDT with 200 l-glutamine	IBA	1.5
11	— " —	NAA	1
12	1/2 × MS (CHENG 1975) with 250 myo-inositol	IBA + NAA	1 0.1
13	— " —	IBA + 2,4-D	1 0.5
14	— " —	IAA + NAA	1 0.5
15	— " —	IAA + 2,4-D	1 0.5
16	CAMPBELL and DURZAN (1975) with 146 l-glutamine and 10 myo-inositol	—	—

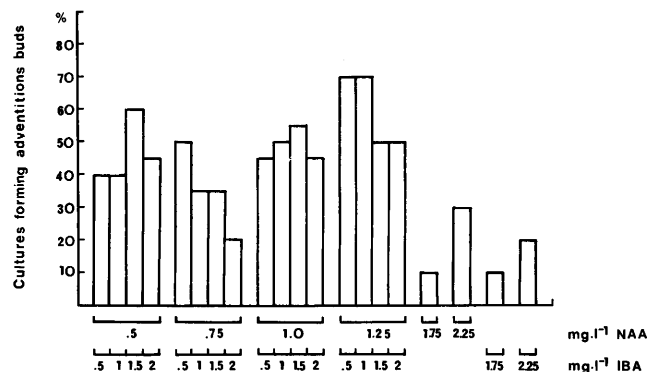


Fig. 1. — Percentage of bud-producing embryos, after 4 weeks in culture, as a function of NAA and IBA concentrations in the medium. Basal medium: 1/2 × MS macroelements, 1 × MS microelements with 3% sucrose, 0.8% difco Bacto-agar, (mg. l^{-1}): 250 myo-inositol, 2 BAP and 2.5 thiamine-HCl. The number of embryos per treatment was 48.

Tab. 2. — Effect of various auxin treatments on three lots of open-pollinated black pine embryos.

Tree	Treatment[a]	mg. l^{-1}	*Bud formation* % of cultures[c]
"47"	NAA	2	45
"221"			30
"47"	NAA +	1	60
"221"	IBA	1	50
"47"	NAA	0.005	55
"221"			60
"47"	NAA +	0.5	30
"221"	IBA[b]	1.5	40
Bulk wild seed lot	Control	0	60—70

[a] Basal medium: 1/2 × MS, 3% sucrose, 0.8% Bacto-agar, (mg. l^{-1}): 2.5 thiamine-HCl, 250 myo-inositol, 2 BAP.

[b] With addition of 10 mg. l^{-1} myo-inositol and 146 mg. l^{-1} l-glutamine. 24 embryos per treatment used.

[c] One excised embryo per culture.

Explants from the 20-day-old seedlings were cultured on a sequence of three modified MS media: 1/2 × MS macroelements, plus 1 × MS microelements supplemented with (mg. l^{-1}): 10 thiamine-HCl, 100 myo-inositol, 1 BAP, 0.001 NAA, 30,000 sucrose and 8,000 agar (A-medium); then, the A-medium without BAP and NAA, used for development of buds (B-medium); then, a B-medium containing 0.3% activated charcoal (C-medium).

The cultures were incubated in a growth chamber at 26 ± 1° C, exposed to artificial light of 700—1500 lx (daylight fluorescent tubes TEŽ-Zgb, 40 W, 220 V, 6,500° K), with light-dark cycles of 16/8 hours.

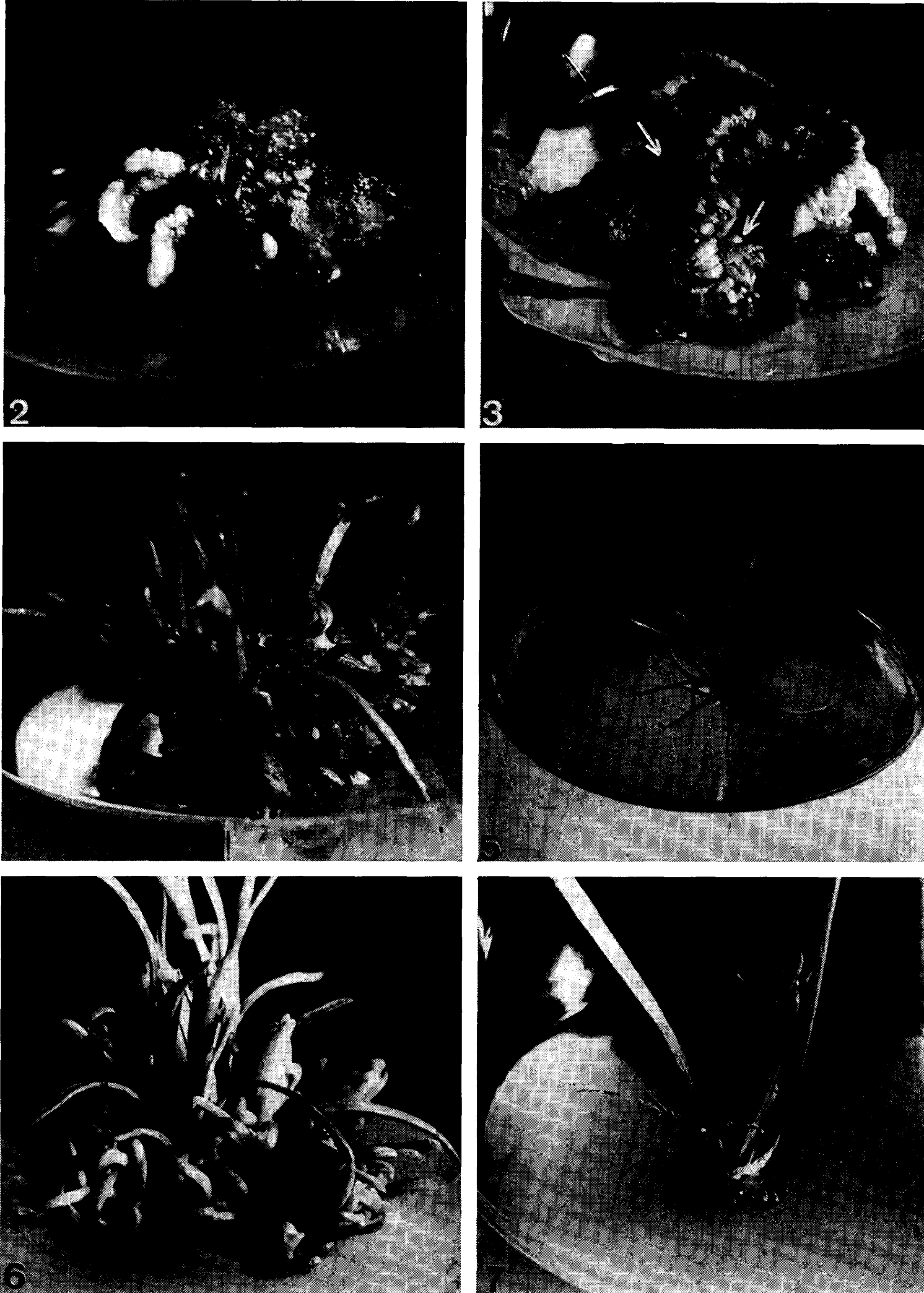
Either 24 or 48 explants were begun in culture for each treatment. Treatments that generated buds were repeated several times.

Results

I. Bud Induction on Embryos

Note that all the media used with excised embryos were supplemented with 2 mg. l^{-1} BAP, but differed in composition of salts, organic constituents and auxins. *Tab. 1* gives details of the various media used. In all of these, more then 50% of the cultures produced callus growth. However, adventitious bud formation (on 20% of the em-

Fig. 2—7. — Generation of buds in *Pinus nigra* juvenile material.



2. Three-week-old embryo cultured on basal medium with (mg. 1^{-1}): 2 BAP + 1 NAA + 0.5 IBA. Bud and needle primordia are generally induced in the meristem part of the shoot apex, as can be seen in the lower central part of this culture (arrow) (x4).
3. Embryo after four-week culture on medium (mg. 1^{-1}): 2 BAP + 1.25 NAA + 1 IBA. Shoots generated on the cotyledon margins of this embryo (arrows).
4. An embryo with induced adventitious buds and needles, three weeks after transferring to hormone-free medium (x4).
5. Adventitious shoot of embryo-culture origin. This shoot was transferred 3 times on the hormone-free medium, at intervals of 4 weeks. Rooting occurred following the third transfer (x1).
6. Second generation of axillary buds on a primary induced shoot formed on an excised seedling shoot tip, on medium with 1 mg. 1^{-1} BAP + 0.001 mg. 1^{-1} NAA (x3.5).
7. Long-shoot development from brachyblast generated from secondary culture, plus new bud (arrow) originating at base of brachyblast, on medium with 1 mg. 1^{-1} BAP + 0.001 mg. 1^{-1} NAA (x4).

bryos) occurred only on a modification of CHENG's (1975) medium containing NAA and IBA together, and in very low percentage (4%) on CAMPBELL and DURZAN (1975) medium without auxin.

The apparently stimulative effect of simultaneously added IBA and NAA in the first set of experiments prompted us to conduct a second set of experiments. In this, a series of combined concentrations of IBA and NAA were evaluated with respect to the induction of adventitious buds. Two levels of IBA and of NAA alone were also included. Results of this second experiment are presented in *Fig. 1*. Media supplemented with a combination of 1.25 mg. l^{-1} NAA and 0.5 mg. l^{-1} IBA showed the highest bud induction, with 70% of cultured embryos producing from one to as many as 55 buds. The other combinations of auxin concentrations were also bud inducing, but with lower efficiency. Concentrations of 1.75 and 2.25 mg. l^{-1} singly added NAA (or IBA) were much less effective than the same summed concentrations of simultaneously added NAA and IBA.

Bud induction with simultaneously added NAA and IBA was repeated in a third set of experiments *(Tab. 2)*, mostly using open-pollinated embryos of two selected trees: "47" and "221". But equal or better bud induction was also obtained in this experiment with singly added NAA in low concentration, and with a control using no added auxin (70%). The latter, however, was achieved using embryos from a bulked seed-lot of wild *P. nigra*.

Buds and shoots generated by cultured embryos always originated from the meristematic region of the shoot apex *(Fig. 2)* or from cotyledon margins *(Fig. 3)*.

Unless explants were transferred to a hormone free medium after about 4 weeks, they were overgrown with callus tissue (see *Fig. 2* and *3* for beginning of such overgrowth) regardless of the medium composition. We did not pursue detailed investigations of the appropriate conditions for extended growth of shoots, but simply noted that their elongation was stimulated by individual transfer to hormone omitted medium *(Fig. 4)*.

Between 0 and 55 buds per embryo were observed in embryo culture of *P. nigra* in the first culture generation (large buds and shoots were counted after the embryos were cultured for 4 weeks on hormone-omitted medium). Some shoots spontaneously rooted while still in the medium *(Fig. 5)*. About 40% of the isolated buds did not survive after being transferred onto fresh medium.

II. Seedling Shoot-tip Culture

New axillary and adventitious buds were induced on the excised shoot tips of 20-day-old seedlings that were cultured on medium supplemented with 1 mg. l^{-1} BAP and 0.001 mg. l^{-1} NAA. Thus, we repeated the procedure used by DAVID *et al.* (1979) for the budding of *P. pinaster* excised shoots. Successive transfers (4 weeks each) of explants from A-medium to B-medium (and sometimes transfers of newly formed shoots to C-medium) yielded additional axillary buds, long shoots and/or brachyblasts *(Fig. 6)*. We have continued this procedure through seven vegetative generations with some of our *P. nigra* clones. In-vitro generated brachyblasts *(Fig. 7)* also produced shoots able to multiply in the same way as those obtained from axillary buds. The buds in the A-cultures that are of apparent adventitious origin require further histological examination to determine their precise origins.

The number of developed shoots per clone varied considerably, and averaged about one shoot per culture per month. However, approximately 30—40% of the newly generated shoots died in the transferring procedure. The highest loss rate was with single shoots transferred to the C-medium, with charcoal.

Discussion

The first experiment led us to think that some combination of high concentrations of IBA, NAA or other auxins was the key to successful tissue culture leading to bud or shoot formation with *Pinus nigra*. The second experiment provided some support for that view with IBA + NAA combinations outperforming high concentrations of either used alone. However, in the third set of experiments, a low concentration of NAA and an auxin-free control did as well or better than the best of the IBA + NAA combinations. We can thus draw two unexpected conclusions at this point. First, the range of auxin concentrations allowing shoot and bud generation is large, from zero to at least 2.25 mg. l^{-1} alone or 3.25 mg. l^{-1} in combination. Second, *Pinus nigra* appears amenable to effective generation of rootable shoots using the procedures outlined above, and additional efforts can reasonably be expended to develop these techniques further with this species.

We have noted several phenomena and problems that now need attention. Included among these are the following. The transfer sequence should be investigated, to reduce the problem of callus overgrowth. Various conditions affecting differentiation of shoots, buds, or other structures (such as needles) are now susceptible to further investigation. The next step in bringing this technique to practical and research uses in forest genetics would be to define procedures for realiably rooting the proliferated shoots, and hardening these plantlets for field experimentation. Then, it can be determined whether shoots from excised embryos (which produce more shoots per culture) are functionally equivalent to shoots from seedling-origin shoot-cultures (which are easier to obtain and to handle), or whether embryo-derived plantlets are more juvenile than seedling-derived plantlets.

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Karyotype Analysis of the Genus Pinus – Subgenus Strobus[1])

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Summary

Descriptions of karyotypes (based primarily on centromere location and arm lengths) are presented for 22 pine species of the subgenus *Strobus* (soft pines). This study concludes a comprehensive investigation of the genus *Pinus* for which karyological information now has been presented for 87 species.

Results for this group of soft pines are similar to those obtained in previous studies. The karyotypes show a remarkable degree of similarity based on general features, yet they are not always identical. Again the karyological data support in general other taxonomic evidence of species relationships, and they indicate the genus has been quite conservative in changing major features of its karyotype.

Important karyological features of the subsections are described and compared.

Key words: Karyotype analysis for 22 pine species of subgenus *Strobus.*

Zusammenfassung

Für 22 Kiefernarten der Sektion *Strobus* werden die Karyotypen erstmalig aufgrund der Lage der Centromeren sowie der Chromosomenschenkellängen beschrieben. Diese Studie schließt eine eingehende Untersuchung ab, aus der nunmehr karyotypische Informationen für insgesamt 87 Arten der Gattung *Pinus* vorliegen. Die Ergebnisse für die Sektion *Strobus* sind denen ähnlich, die in früheren Untersuchungen erzielt wurden. Die Karyotypen zeigen einen bemerkenswerten Grad an Ähnlichkeiten bei den Hauptmerkmalen, sie sind aber nicht immer identisch. Wiederum unterstützen die karyotypischen Daten im allgemeinen andere taxonomische Merkmale der Verwandtschaft zwischen den Arten und zeigen an, daß die Gattung bei der Änderung von Hauptmerkmalen der Karyotypen sehr konservativ gewesen ist. Wichtige Karyotypmerkmale der Untersektionen werden beschrieben und verglichen.

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Introduction

Although previous studies have shown that the karyotypes of pine species are in general similar, differences have been observed that are useful in understanding relationships among some of the species. Based primarily on centromere location, SAYLOR (1964, 1972) found differences between certain subsections of hard pines (subgenus *Pinus)* and occasionally between species. PEDERICK (1967, 1970) also illustrated interspecific differences in his detailed karyotypic analyses based on the size and location of secondary constrictions.

Karyotypes are described in this paper for 22 species of soft pines (subgenus *Strobus)*[3]). This report includes data for a majority of the species in this subgenus, and concludes the comprehensive karyological study of the genus *Pinus* on which 87 species have now been reported.

Materials and Methods

Karyotype data for this and previous studies were obtained by using identical procedures. Slides were prepared from squash preparations of root-tip meristerms of 2—12 month old seedlings. The root-tips were pretreated in oxyquinoline (0.3 g/l.) for 24—36 hours at 12° C, fixed in 3:1 ethyl alcohol-acetic acid for 1—4 hours, hydrolyzed in 1N HCl for 10—15 minutes at 60° C, and stained in acetocarmine.

Chromosome dimensions were obtained from projection drawings as described by SAYLOR (1961). To allow comparisons, the data are presented in the same manner used in previous reports. For each karyotype, the chromosomes are arranged from 1—12 according to a descending order of lengths of the short *(a)* arms. (Chromosomes with median and submedian centromeres are defined by short-arm/long-arm ratios of 0.75—1.00 ınd 0.50—0.75, respectively.)

The diagnostic features used in making species comparisons included:

[3]) The taxonomic classification used in this presentation follows that of CRITCHFIELD *et al.* (1966) and LITTLE *et al.* (1969).