Recent Advances in the Study of Apomixis in Juglandaceae

Guo-liang Wu1,2*, Yu-qin Song3, Jaime A. Teixeira da Silva3, Qun-long Liu4, Lan Ji2, Jun-qiang Yang1, Peng-fei Zhang5, Jin-he Bai6

1 College of Forestry and Horticultural Science, Henan Agricultural University, Zhengzhou, 450002 Henan, China
2 College of Horticultural Science, Shanxi Agricultural University, Taiyuan, 030081, Shanxi, China
3 Department of Horticultural Science, Faculty of Agriculture, Kagawa University, Miki-cho, Ikenobe, Kagawa-ken, 761-0795, Japan
4 Institute of Horticultural Science, Shanxi Academy of Agricultural Science, Taiyuan, 030001, Shanxi, China
5 Agricultural High Technological School of Xinyang, Xinyang, 464000, Henan, China
6 Mid-Columbia Agricultural Research and Extension Center, Oregon State University, Experiment Station Drive, Hood River, 3005, OR, USA

Corresponding author: * walnut-wu@126.com

ABSTRACT
Walnut, Juglans regia L., is the most economically important member of the Juglandaceae family and is the most valuable nut crop in the world. Recently, it has increased greatly in acreage and in production. Although a number of research programs about plant apomixis have been carried out around the world, few were represented by the Juglandaceae family. This paper presents a brief review of the current status of knowledge in the field of plant apomixis research, especially in walnut, including chemical induction, bagged isolation, observation of embryology, analysis of isoenzymes, physiological and biochemical characteristics of apomictic seedlings. We further introduce a strain which may be an obligate apomictic walnut, and discuss the problems associated with and the developmental prospects and progress in this field.

Keywords: bagged isolation, embryology, isoenzyme, new strain, walnut (Juglans regia L.)
Abbreviations: BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; Kn, kinetin; LH, lactalbumin hydrolysate; POD, peroxidase; DKW, Driver and Kuniyuki for walnut culture medium

CONTENTS
INTRODUCTION .......................................................................................................................... 5
THE CONCEPT AND SIGNIFICANCE OF PLANT APOMIXIS .......................................................... 5
Concept ...................................................................................................................................... 6
Classification .............................................................................................................................. 6
Recent progress .......................................................................................................................... 6
Methods to determine apomixis ................................................................................................. 6
RECENT ADVANCES IN THE STUDY ON APOMIXIS IN JUGLANDACEAE ............................................ 6
Chemical induction .................................................................................................................... 6
Isolation studies ........................................................................................................................ 7
Study of bagged isolation ........................................................................................................... 7
Mentor pollen isolation study .................................................................................................... 7
Embryologic studies .................................................................................................................. 7
Studying the features of apomictic walnut seeds ....................................................................... 7
Germination of seeds ................................................................................................................ 7
Reaction of young embryos under tissue culture of walnut seeds ................................................ 7
Germination of seeds ................................................................................................................ 7
Growth characteristics of apomictic seedlings ......................................................................... 7
Isoenzymes to study walnut apomictic seedlings .................................................................... 7
Study on the selection of new strains with apomictic traits in walnut ...................................... 8
CONCLUSIONS ........................................................................................................................ 8
ACKNOWLEDGEMENTS .......................................................................................................... 8
REFERENCES ............................................................................................................................ 8

INTRODUCTION
Some Juglandaceae plants are important cultivated forms and have economic value in the world. They belong to the Juglans and Carya genera. The major forms in China include Juglans regia L., J. sigillata Dode., J. cathayensis Dode., J. hopeiensis Hu, J. mandshurica Max., J. nigra L., Carya cathayensis Dode., and C. ilicifolia (Wangen.) K. Kohn. In China, walnut (Juglans regia L.) has the top growth area (reached 1300 thousand hm² in 2000 and output reached 300.98 thousand t), and there exist more than 4000 cultivars in China (Xi et al. 2005). Walnut originated from south-eastern Europe and western Asia and apomictic traits were found many years ago (Loiko 1990; Asadian et al. 2005). In fact, apomixis produces seeds through asexual processes. The genetic make-up of the seeds is identical to that of the mother plant. If the mother plant is well adapted to a particular environment or purpose, so will be the offspring (Jefferson 1994; Spillane et al. 2001a). Accordingly, to make use of apomictic walnuts to breed seed-
lings will provide a new way to extend improved cultivars for walnut production (Koltunow 2003).

The selection of varieties with obligate apomixis reduces the effect of bad climatic conditions on yield, and is especially important in the breeding of new varieties in cold regions (Loiko 1990; Valdiviesso 1990) where lower temperature and strong wind would limit the pollination badly.

This paper reviews studies on apomixis in walnut and other related species in order to provide some insight into walnut research and production.

THE CONCEPT AND SIGNIFICANCE OF PLANT APOMIXIS

Concept

The term apomixis was formerly used as a synonym of vegetative propagation. At present, it is preferentially restricted to the sense of agamospermy, i.e. asexual reproduction by seeds (Jefferson 1994; Sun et al. 1996). In apomictic reproduction, the embryo develops autonomously from an unreduced cell having the same set of maternal chromosomes and giving rise to plants that are clones of the mother plant (Sun et al. 1996; Carneiro et al. 2006).

Classification

Apomixis is a wide-spread phenomenon in the plant kingdom present in more than 36 families of Angiosperms (Hanna and Bashaw 1987; Pichot et al. 2001). According to the degree of apomixis, the process can be classified into two types: obligate and facultative. In obligate apomixis, there is no fusion of male and female gametes and the egg cell develops autonomously, by parthenogenesis, generating an embryo that keeps the same set of maternal chromosomes and leading to the formation of embryos genetically identical to the mother plant.

In facultative apomixis, the plant can reproduce both sexually and asexually, leading to progeny plants with new features. These are desired traits during plant breeding to generate new genotypes better adapted toiotic and abiotic environmental factors.

There is no clear-cut separation between the two types. With a few exceptions it has been observed that apomictic species are polyploid, while the sexual varieties of the same species are diploid (Dong et al. 1996; Zhou et al. 1998). It is known that most apomicts are facultative apomixis (Sun et al. 1996; Gao et al. 2000; Carneiro et al. 2006).

According to the origin of apomictic generants, apomixis can be aposporous initial: three types: diplospory, aposporous and adventitious embryogenesis. In diplospory, there are two forms (mitotic and meiotic) during gametophytic development, the megaspore-mother cell does not enter meiosis or is arrested at an early stage during meiosis and only undergoes mitotic divisions without a reduction of the genome. In both forms, an embryo sac is formed consisting of an unreduced, diploid egg cell (Leblanc et al. 1995). In aposporous, initial cells form from nucellar cells and differentiate after three mitotic divisions into embryo sacs containing a diploid egg cell that directly develop into an embryo without fertilization. This type is common in the Poaceae. Adventitious embryogenesis initiates from the somatic tissues of the ovule, nucellar or integumental cells. These cells developed directly into embryos and compete with the sexual embryo that is formed after fertilization. This type is lacking in the Poaceae but is common in other families (Koltunow 1993; Leblanc et al. 1995; Ma et al. 2001).

Recent progress

It was always thought that apomixis occurred in all Angiosperms, although only few reports occurred in woody plants, mainly in Malus spp. (Dong et al. 1996), walnut (Schanderl 1964), Xanthorrhium boraeum (Liu Y-H et al. 1987), orange (Citrus) (Cai et al. 1997) brumble (Rubus alcefolius) (Amsellem et al. 2001) and so on. Recent studies showed that a new species in the Clusiaceae, Hypericum carpathianum demonstrated apomixis, and that it may be a hybrid of H. maculatum × H. perforatum, with features from the paternal H. perforatum (Marionfi 2001). Newly discovered Cotoneaster spp. (Rosaceae), such as Cotoneaster brickellii, C. latifolius, C. purpurescens, C. vandeliariae, C. thimphuensis, C. sieversii, C. lancasteri, C. kingdonii, C. shannanensis and C. yui, had apomictic feature (Fryer and Hylmo 2001; Bartish et al. 2001). All plants in the Rosa section Caninae were polyplody and apomicts (Werlemark 2000; Schranz et al. 2005).

Methods to determine apomixis

At present there are many methods to study and identify plant apomixis, with few comprehensive reviews (Ma et al. 2002; Carneiro et al. 2006). Ways to study the apomictic characteristics of walnut include induction with chemicals (Xi et al. 1996), investigation of the setting ratio by bagging isolation (Loiko 1990; Valdiviesso 1990; Gao S-T et al. 1999; Wang G-A et al. 2003; Ghavamaldin et al. 2005; Bekir et al. 2006a, 2006b), observation of the embryo origin and developmental stage by embryology (Chen Y-F et al. 2000; Yang et al. 2006; Wu G-L et al. 2007), analysis of biochemical characteristics by isoenzymes, observation of morphological features of nuts, and investigation of the germination and morphological features of the apomictic seedlings (Wang G-A et al. 2003; Yang J-Q et al. 2006; Wu et al. 2007).

To determine the rate of apomixis, a number of walnut female flowers were isolated and after three weeks bags were removed and flowers counted and recounted after 14 days again. During harvest the number of fruits was also counted. Cultivars having fruits in the bags were recognized as cultivars with apomixis. The degree of apomixis was determined as the number of fruit calculated to the number of isolated flowers.

The degree and capacity of apomixis was also determined as the number of fruit calculated to the number of open-pollination flowers (Zhang et al. 2000). Different standards in studying walnut apomixis by different researchers can result in difficulties coordinating research. Consequently, since most researchers defined apomixis as the number of fruits relative to the number of isolated flowers in walnut, we also adopted this definition in this review.

RECENT ADVANCES IN THE STUDY ON APOMIXIS IN JUGLANDACEA

The study of apomixis in Juglandaceae plants involved intra- and interspecific Juglans spp., such as J. regia L., J. nigra L., J. mandshurica Maxim., J. cinerea L., J. sieboldiana Maxim. and two interspecific hybrids (Badalov 1983), however, most of them were about walnut (J. regia L.).

Chemical induction

Chemically-induced apomixis is rare, and the only report was from Hebei Agricultural University of China, in 1962-1963 using 2,4-D and other chemical. The fruit set ratio treated with 2,4-D at 10-30 mg/l was 3.2%-18.5% (Xi R-T et al. 1992).
Isolation studies

Presently, there are two ways to isolate walnut female flowers: one is by using bags made of a sleeve of paper to implement spatial isolation; the other is by artificial fertilizing the chaplet or mentor with several kinds of plant pollen and then blocking it.

Study of bagged isolation

Reviewing the literature related to walnut apomixis, it can be seen that the main method of researching walnut apomixis has been to isolate the female flowers with bags, so that parthenocarpy can be artificially induced.

In general, Juglandaceae plants (with few exceptions) display a certain frequency of apomixis despite the different cultivars, different years and different regions. A comparison of plants from the same region and of the same cultivar but from different years showed great differences in their apomixis features.

From Table 1 to 3 the data illustrates an irregular presentation of apomixis within the same region and over different years: some cultivars did not demonstrate apomixis over two successive years, like ‘Liaohe No. 3’ (Yangling, Shannxi, China); some cultivars changed a little in their capacity to develop apomixis, like ‘Taishan No. 2’ (Taian, Shandong, China), ‘Zhonglin No. 1’ and ‘Liaohe No. 1’, but others changed irregularly. This phenomenon is mainly affected by environmental factors, especially air temperature in the different years, and all these plants are facultative apomicts. It is noticeable that cv. ‘Zuoquanmian’ had the highest percentage of apomixis in three years, especially in 2003 when other cultivars did not demonstrate apomixis, and its apomictic behaviour was steady. This cultivar is considered to be obligate apomict, which is in agreement with former conclusions (Loiko 1990).

The method that uses spatial isolation with bags after emasculation prevents pollen from reaching the female flowers. They are bagged approximately a week before pistillate blooming in walnut (Loiko 1990; Valdiviesso 1999). However, when pistillate flowers of walnut in bags were pollinated with Chinese pine (Pinus tabulateformis) pollen, there was almost no mentor effect in the rate of apomixis (Liu D-L et al. 1999). The results of a study conducted in Turkey showed that the pistils of 5 genotypes (Yalova-1, Bilecik, 07-KOR-1, Tokat-1 and Kaman-1) that had apomictic fruit set could be pollinated with freshly collected pollen of apple cv. ‘Golden Delicious’ (Malus domest Borkh.), and the percentage of apomictic fruit set ranged from 0.5% to 1.9%. As a result of pollination with apple pollen, open pollination set 37% (CK), while the highest apomictic fruit set was 6.4% (“Tokat-1”) (San et al. 2006b).

Embryologic studies

Paraffin sections are an effective way to systematically observe the dissectional construction of the plant ovule. It allows one to observe the position of the gametes, megasporogenesis and formative process of the embryo sac, and to distinguish the phenomenon of meiosis and morphological characters of the ovule and other cells. These characteristics were the key to identifying apomixis in plants (Li et al. 1992; Ma S-M et al. 2002).

In earlier work, Schanderl suggested that the embryo obtained from apomixis was not formed in the place where the embryonic sac was usually found but rather situated near the micropyle, always in the area between the micropyle and the nucellar cavity. Sometimes it is formed very close to the micropyle or a bit far apart from it, but never more than 13 tiers of cells, from a nucellar somatic cell (Schanderl 1964).

The result of Chen Y-F (2000) showed that there was an

Table 1 The nut setting in walnut without pollination (Yangling, Shannxi, China) (from Gao S-T et al. 1999, with permission).

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Female flowers</th>
<th>%</th>
<th>Female flowers</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xinlin No. 3</td>
<td>54</td>
<td>5.6</td>
<td>135</td>
<td>2</td>
</tr>
<tr>
<td>Liaohe No. 1</td>
<td>23</td>
<td>4.3</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>Liaohe No. 2</td>
<td>73</td>
<td>6.8</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Xinfu No. 1</td>
<td>32</td>
<td>0</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>Xiangling</td>
<td>7</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2 The nut setting in walnut without pollination (Taian, Shandong, China) (from Zhang et al. 2000, with permission).

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Female flowers</th>
<th>%</th>
<th>Female flowers</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xingling</td>
<td>230</td>
<td>11.3</td>
<td>150</td>
<td>17</td>
</tr>
<tr>
<td>Fenghui</td>
<td>230</td>
<td>9.1</td>
<td>180</td>
<td>17</td>
</tr>
<tr>
<td>Luguang</td>
<td>200</td>
<td>9.0</td>
<td>210</td>
<td>28</td>
</tr>
<tr>
<td>Yuanfeng</td>
<td>160</td>
<td>13.1</td>
<td>230</td>
<td>33</td>
</tr>
<tr>
<td>Liaohe No. 1</td>
<td>110</td>
<td>13.6</td>
<td>180</td>
<td>28</td>
</tr>
<tr>
<td>Lufeng</td>
<td>150</td>
<td>13.3</td>
<td>190</td>
<td>18</td>
</tr>
<tr>
<td>Zhonglin No. 1</td>
<td>250</td>
<td>9.2</td>
<td>230</td>
<td>31</td>
</tr>
<tr>
<td>Taishan No. 1</td>
<td>350</td>
<td>12.0</td>
<td>150</td>
<td>18</td>
</tr>
<tr>
<td>Taishan No. 2</td>
<td>270</td>
<td>14.1</td>
<td>250</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 3 The nut setting in walnut without pollination (Taing, Shannxi, China) (from Wu et al. 2007, with permission).

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Female flowers</th>
<th>%</th>
<th>Female flowers</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhonglin No. 1</td>
<td>53</td>
<td>15.1</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>Liaohe No. 1</td>
<td>39</td>
<td>5.1</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>Taigu Mian</td>
<td>123</td>
<td>6.5</td>
<td>142</td>
<td>0</td>
</tr>
<tr>
<td>Zuoquan Mian</td>
<td>131</td>
<td>51.1</td>
<td>103</td>
<td>8</td>
</tr>
</tbody>
</table>
intact and typical Polygonum-type of megaspore in three walnut cultivars (Xilin No.3, Liaohe No.1 and Xifu No.1), and the embryo derived from apomixis expectedly came from egg cells and remained parthenogenic. The egg cell of the embryo sac in apomixis began to cleave after a short period of dormancy (about two weeks) from a two-cell, four-cell, eight-cell up to a globular embryo, developed more and became a multicellular embryo (Fig. 1; Chen Y-F et al. 2000). Our recent results confirm this process of apomixis in walnut (Wu et al. 2007).

Studying the features of apomictic walnut seeds

Germination of seeds

Reaction of young embryos under tissue culture of walnut seeds

The percentage of germination of apomictic seeds was generally low, the amount was very poor and apomictic seeds could not always be obtained. However, somatic embryogenesis from immature cotyledons of a few apomictic seeds could provide the possibility of mass clonal propagation of cultivars in walnut species that are difficult to use in vitro techniques such as shoot tip culture.

In a study of somatic embryogenesis, explants from immature cotyledons of seeds thought to be of apomictic origin of 4 genotypes were cultured 8 weeks after anthesis on an initial medium consisting of DKW (Driver and Kuniyuki,1984) medium for 3 weeks: DKW + 1 mg l⁻¹ BA + Kn 2 mg l⁻¹ + 0.01 mg l⁻¹ IBA + 250 mg l⁻¹ LH; then subculture on DKW + 0 (i.e. hormone-free) for four weeks; the percentage of cotyledons that formed embryos ranged from 3.6% to 25%, and secondary embryogenesis from somatic embryo explants progressed well (San et al. 2006a).

Germination of seeds

The germination of apomictic seeds is usually lower than seeds from open-pollination, averaging 74.1%, far less than from open-pollination (96.8%; see Table 5). This phenomenon showed that embryo development was poor.

The cotyledons of apomictic walnut seeds developed poorly, frequently lacking a quarter or half the cotyledon. From Tables 4 and 5 it can be seen that the germination of apomictic walnut seeds depends on the year, the average from 23.0%, 54.4% to 43.7% (see Table 4), but it was lower than CK (96.8%) (Loiko 1990; Wang G-A et al. 2003; Yang 2006).

There was a high rate of empty nuts (with a hardy shell and seed capsule but no cotyledon) in walnut nuts derived from apomixis following harvest, the average of two years reaching 7.1% while only 0.5% of nuts were derived from natural pollination (Table 5). The emergence rate (the ratio of walnut seed number to the walnut seedling number in the apomixes) of walnut arising from apomixis was relatively low with an average value of 74.1% in two years in Taigu and Shanxi (locations of tests: regions in north China) while 96.8% of walnuts resulted from natural pollination, outclassing apomixis.

A lot of walnut seeds developed incompletely with a quarter to half of the cotyledon missing following examination of apomictic walnuts. The spherical embryo appeared at a later stage in apomictic walnut than from natural pollination and the apomictic embryos developed slowly, which may explain why nuts are empty or have a shriveled kernel when the walnut matures. Besides slow embryo development, the apomictic embryo also developed the endosperm poorly and the delay in its development also resulted in the incomplete development of matured nuts.

The main likely reasons for the degeneration and halted development of apomictic seeds were likely: i) there was no natural apomictic megaspore formed in the ovule; ii) the apomictic embryo degenerated in mid-development for

Fig. 1 Apomictic embryology of walnut showing the non-pollination embryo sac and embryo development. 1, egg cell, two polar nucleus (PN) and three antipodals (AT), ×800; 2, two-cell embryo (EM), ×550; 3, four-cell embryo (EM), ×550; 4, eight-cell embryo (EM), ×550; 5, the polar nucleus where fusion occurs, ×600; 6, three endosperm cells that split (EN), ×1000. (adapted from Chen Y-F et al. 2000 and Wu G-L et al. 2007, with permission)
zymes are the product of gene expression, and thus isoenzyme zymograms of offspring originating from apomixis were baccata to their seedling progenies with high heterozygosity. great difference among individuals with the segregation of characters in heterozygosis did not exist in seed development. In the control, there was a difference in apomictic offspring did not involve the combination of male and female gametophytes and that the segregation of characters in heterozygosis did not exist in seed development. In the control, there was a difference in apomictic offspring did not involve the combination of male and female gametophytes and that the segregation of characters in heterozygosis did not exist in seed development.

Table 4 The germination of walnut nuts having apomixis (Wensu, Xinjiang, China) (from Wang G-A et al. 2003, with permission).

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>1999</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. seeded</td>
<td>%</td>
</tr>
<tr>
<td>Zha343</td>
<td>15</td>
<td>11.7</td>
</tr>
<tr>
<td>Xinzaofung</td>
<td>14</td>
<td>11.7</td>
</tr>
<tr>
<td>Xinxin2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Xinwen81</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Xinwen179</td>
<td>46</td>
<td>6</td>
</tr>
<tr>
<td>Xinwen185</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>Xinfung</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>Xincunfeng</td>
<td>34</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>186</td>
<td>32</td>
</tr>
<tr>
<td>Average</td>
<td>23.0</td>
<td>54.4</td>
</tr>
</tbody>
</table>

Table 5 Germination rate of nuts from different walnuts (from Yang 2006, with permission).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Apomixis seedlings</td>
<td>6.7 (6.89)</td>
<td>7.4 (2.27)</td>
<td>7.1</td>
<td>72.3 (60/83)</td>
<td>76.0 (19/25)</td>
<td>74.1</td>
</tr>
<tr>
<td>Progeny from open-pollination</td>
<td>1.0 (1/100)</td>
<td>0 (0/30)</td>
<td>0.5</td>
<td>97.0 (96/99)</td>
<td>96.7 (29/30)</td>
<td>96.8</td>
</tr>
</tbody>
</table>

Table 6 Plant height of different biennial walnut seedlings (from Yang 2006, with permission).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Progeny from apomixis</td>
<td>60</td>
<td>51.4</td>
<td>12.4</td>
<td>0.2</td>
<td>3.38</td>
<td></td>
</tr>
<tr>
<td>Progeny from open-pollination</td>
<td>72</td>
<td>62.4</td>
<td>24.0</td>
<td>0.4</td>
<td>(u: 0.01=2.58)</td>
<td></td>
</tr>
</tbody>
</table>

Table 7 Isoenzyme zymogram of POD from apomictic seedlings (from Wu et al. 2007, with permission).

<table>
<thead>
<tr>
<th>Source of seedling</th>
<th>Zymogram appearance percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progeny from apomixis</td>
<td>Rf=0.38</td>
</tr>
<tr>
<td>Progeny from open-pollination</td>
<td>Rf=0.29</td>
</tr>
</tbody>
</table>

being uncompetitive in absorbing and translocating nutrition.

Growth characteristics of apomictic seedlings

Seedlings derived from apomixis were significantly (P = 0.01) shorter than those from natural pollination (Table 6). This indicated a great trend in reduction of walnut seedling height from apomictic than from natural pollination. The coefficient of variation in plant height was 0.2 in the former and 0.4 in the latter, and expected result that would explain the lower degree of separation in apomictic seedlings than those derived from sexual reproduction.

The apomictic seedlings performed uniformly, which may be due to the fact that apomictic offspring did not involve the combination of male and female gametophytes and that the segregation of characters in heterozygosis did not exist in seed development. In the control, there was a great difference among individuals with the segregation of character in their seedling progenies with high heterozygosity of the seeds originating from crosses.

Isoenzymes to study walnut apomictic seedlings

Isoenzymes were used to study plant apomixis since this method is quick, cheap and generates considerable polymorphism, and has already been used widely in orchard fruit apomictic studies. Five-month old orange seedlings were studied by isoenzyme analysis of phosphoglucoisomerase and phosphoglucomutase in the lamina. The isoenzyme zymogram indicated a clear distinction between zygotic and adventitious embryos that originated from the nucellar embryo cell (Ellisario et al. 1999). According to Liu H-Z (1999), apomicts of Malus sikkimeniis had a relatively highly uniform peroxidase isoenzyme zymogram besides showing developmental uniformity and parthenocarpy. Results of this study showed that the percentage banding pattern of M. sikkimeniis apomictic seedlings was above 80%, but 100% in Malus xiaojinensis and 50% in Malus baccata L. as controls, which showed that germplasm homozygosity of offspring originating from apomixis was higher than that originating from sexual reproduction. Enzymes are the product of gene expression, and thus isoenzyme zymogram uniformity showed direct proof of germplasm homozygosity. Plant isozymes have been a good biochemical index to study plant development and cell differentiation due to their tissue and phase specificity (Kuhns et al. 1978; Cheng S-Z et al. 1987; Dong S-Z et al. 1989).

We studied peroxidase isoenzyme using the lamina of apomicts and common walnut seedlings. The results showed two clear banding patterns in the test (Table 7; Fig. 2: Wu G-L et al. 2007). The performance frequency of the peroxidase isoenzyme zymogram (POD) among individual plants was used to calculate the banding pattern performance percentage while its mean value represented the uniformity. In this test, 'Liaohe No. 1' seedlings from a natural pollination as the control had an isoenzyme zymogram uniformity of 55% but 86% in apomictic seedlings.

The results showed that isoenzyme zymogram uniformity of POD in apomictic seedlings was higher than the control. This indicated that the heredity background uni...
formity of apomictic seedlings was higher than seedlings derived from control seeds, and that its characters developed uniformly with a little degree of separation (Wu et al. 2007).

**Study on the selection of new strains with apomictic traits in walnut**

The selection of varieties with obligate apomixis decreases the effect of unfavourable climatic conditions on yield (Loiko 1990). Different forms of walnut with apomixis can be used in hybridization, so this has great significance in introducing walnut to regions of severe climatic conditions. There are few studies about variety selection in apomictic walnut, but no reports on the selection of new cultivars (Badalov 1983). On the basis of researching apomixis walnut, we managed to make a new cultivar selection and found a single cultivar which combined economic characteristics, such as single fruit weight and yield per m², and also had apomictic ability (Table 8; Wu et al. 2007). Selected forms having regular apomixis are referred to as obligate apomicts (Loiko 1990).

**CONCLUSIONS**

Walnut, *Juglans regia* L., is the most valuable nut crop in the world and the most economically important member of the genus *Juglans*. Recently, it has increased greatly in acreage and in production around the world. Although a number of plant apomixis research programs have been carried out around the world, few are dedicated to walnut. Allogamic biological features which are restricted by environmental conditions in walnut also affect output badly. Over the years it has been one of the aims to seek to breed new cultivars by using apomixis.

Though there have been many papers and reviews about apomixis (Bartish et al. 2001; Grossniklaus et al. 2001; van Baaren et al. 2002; Wang Z-W et al. 2004; Carneiro et al. 2006), basically they were limited to herbaceous, monocotyledonous plants (Sun et al. 1996; Gao J-W et al. 2000; Akiyama et al. 2004; Albertini et al. 2004). Among woody fruit trees most studies were related to *Malus* spp. (Dong S-Z et al. 1987, 1989; Liu H-Z et al. 1989; Zhou Z-Q et al. 1995; Dong W-X et al. 1996; Zhou et al. 1998) while apomixis in other fruit trees were scarcely reported (Liu Y-H et al. 1987; Cai et al. 1997, 2002; Nybom 2004; Carneiro et al. 2006). Over 60 years of studying walnut apomixis we now know that there are two types of apomixis, obligate and facultative in walnut, following the observation and analysis of morphological features. Using these characteristics to breed new varieties may enhance the adaptability of this important nut crop and obtain steady output.

The walnut tree is a perennial woody plant, large and with many phenols in its tissues, which causes some problems in research related to increasing the growth cycle, difficulties in sampling and in experimenting. At present in depth research is limited. The mechanisms underlying recombination and the genetic regulation of apomixis in walnut are far from being clarified.

Along with the advances in biotechnology, an interest in apomixis has increased, and the study of apomixis has deepened to the molecular level in most crop plants (Jefferson 1994; Grossniklaus et al. 2001; Bartish et al. 2001; Ma S-M et al. 2002; Eckardt 2003; Akiyama et al. 2004; Albertini et al. 2004; Chen and Guan 2006). The study of apomixis ultimately seeks a regular pattern or mechanism to control the expression of genes responsible for apomixis. This would allow the transmission of the male progenitor characteristics to the progeny (Spillane et al. 2001b; Albertini et al. 2004; Carneiro et al. 2006).

Many of the results obtained so far would promote research of the mechanisms of apomixis in walnut, many of which will contribute to programs aimed at the generation of improved and asexually seed-propagated walnut (Spillane et al. 2001b; Albertini et al. 2004). Using novel breeding strategies, combined with novel genetic, molecular and cytological methods it is now possible to attempt to understand the genetic regulation of sexual and asexual ways of reproduction for utilizing apomixis in agriculture and walnut improvement in the near future.

**ACKNOWLEDGEMENTS**

This work was supported by the Research Foundation of Science and Technology of Shandong Province, Project No. 021036; the China National Natural Science Foundation, Project No. 30571290, the Shandong Province Natural Science Foundation, Project No. 2006011081. The authors thank Dr G X Chen, the professor and the library curator of Shandong Agricultural University; Dr D Y Zhu, the professor and the vice-president of Henan Agricultural University; Ms. Li-fang Cheng, the associate professor of Shandong Agricultural University for their kind help.

**REFERENCES**

*A* In Chinese


Carneiro VTC, Dusi DMA, Ortiz JPA (2006) Apomixis: occurrence, applica-

* tions and improvements. In: Teixeira da Silva JA (Ed) Floriculture, Orna-


t
nemis. Journal of Fruit Science 6, 103-105*


Jefferson RA (1994) Apomixis: A social revolution for agriculture. The Center for the Application of Molecular Biology to international Agriculture (CAM BIA), Biotechnology and Development Monitor 19, 14-16


Koltunow AM (1993) Apomixis: Embryo sacs and embryos formed without meiosis or fertilization in ovules. The Plant Cell 5, 1425-1437


Sun B, Humanglu H (2006a) Determination of the apomictic fruit set ratio in some Turkish walnut (Juglans regia L.) genotypes. Turkish Journal of Agricultural Forestry 30, 189-193


Schanjdel R (1964) Untersuchungen über die blutenbiologie und embryonen- bildung von Juglans regia L. Botanisches Zentralblatt 83, 71-110


Yang J-Q (2006) Study on physiological and biochemical characteristics of apomictic walnut seedlings. MSc Thesis, Shanxi Agricultural University, China*

