Research Report

PLANT REGENERATION FROM WALNUT PROTOPLASTS, ENDOSPERM AND EMBRYOS

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Abstract

The objective of this project is to develop the techniques and capability to regenerate plants from specific walnut cell and organ sources. This is an essential step toward the utilization of novel breeding and selection procedures for walnut improvement, especially disease resistance. Embryonic axes from Juglans regia cultivars, J. hindsii, and Paradox have been dissected out and raised to young plants. In addition, the procedures needed to grow immature embryos from genetic crosses have been developed. This rescue of embryos is part of the effort to improve walnut rootstock for Phytophthora resistance.

Embryo-like structures (embryoids) have been induced by growing walnut cotyledon tissue on a specific sequence of nutrients. However, a reliable system for the regeneration of plants from embryoids remains to be worked out. The results, thus far, suggest that embryoids may be derived from leaves and might therefore be useful in a screening program for disease resistance based on somatic genetic variants.

The many steps required for the isolation of viable protoplasts from walnut leaves are under investigation. Protoplast preparations have been obtained, but the techniques must be refined. Regeneration from such preparations also needs to be worked out.

The endosperm tissues of several walnut cultivars, J. hindsii and Paradox have been removed from seeds and placed on a variety of nutrient media, but the tissue has failed to proliferate. It is expected that utilizing the correct stage of development and a wider range of nutrients will lead to tissue growth. Plants derived from endosperm would have a unique genetic base which would be useful for rootstock selection.

OBJECTIVE

The objective of this project was to develop the techniques for plant regeneration from walnut protoplasts, endosperm and embryo. These techniques will be an integral part of the Walnut Breeding Program which will involve the manipulation and selection of plants in culture, especially for disease resistance. The advantage of regenerating plants with these techniques is that it allows the recovery of plants which are somatic variants, thus providing more diversity for screening and selection procedures. It is also an essential step for later work on protoplast fusion and genetic engineering.
PROCEDURES

1. Embryo
   a) Embryos from crosses of Pterocarya stenoptera x Juglans regia were dissected out from surface sterilized fruits and grown on nutrient media. This procedure of embryo rescue is designed to save embryos which might not otherwise survive.
   
   b) Embryonic axes (embryos with the cotyledons removed) were dissected out from surface sterilized fruits of Payne, Hartley, Scharsch-Franquette, Amigo, Tehama, Chico, J. hindsii and Paradox. These embryonic axes were grown on many experimental media in order to determine the medium which would support embryo growth the best. The media of Driver and Kuniyuki (DKW, unpublished), Murashige and Skoog, Cheema and Sharma, and others were used. Growth regulators (benzylaminopurine, indole butyric acid, naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, gibberellic acid, and kinetin), nitrogen sources (casein hydrolysate, proline, glutamine) and other organic compounds (spermine, catechin, phloridzin) as well as charcoal were tested for their effect on embryo development.
   
   c) Cotyledons, leaves, roots, cambium and petiole were grown in culture on various media mentioned above. The purpose was to induce callus growth, differentiation and somatic embryogenesis.

2. Endosperm
   Walnut fruits from Payne, Hartley, Chico, Tehama, Scharsch-Franquette, Sinensis, and J. hindsii were collected from July-September, 1983, surface sterilized, and the available endosperm tissue was dissected out and cultured. A similar variety of plant growth regulators, nitrogen sources and other constituents were used in media. In addition, the medium of Murashige and Skoog, reported by Cheema and Mehra to promote the growth of endosperm tissue of walnut, was tried in a factorial experiment with kinetin and naphthalene acetic acid.

3. Protoplasts
   Callus suspension cultures of J. hindsii were filtered through Nitex screens, partly plasmolyzed in a hypotonic 0.6 M solution of mannitol and nutrient medium for one-half hour, then placed in digestive enzymes: Macerase (0.5-1.0%) to loosen the cell clumps and cellulase (2.0-2.5%) to erode the cell wall. Digestion of the cell wall was followed by Calcoflor
stain and visualization with ultraviolet microscopy. Viability of the protoplasts was determined by fluorescein diacetate and microscopic examination. Osmoticum and enzymes were washed from the cells by appropriate centrifugation and all procedures were done to maintain sterile conditions. Protoplasts were suspended in isotonic nutrient media and placed in culture for cell wall regeneration and callus formation.

RESULTS

1. Embryos

a) Embryos from Pterocarya crosses have been grown in culture to small plants. The procedures used were successful and should be useful for wide crosses in the future.

b) The embryonic axes of many cultivars of Juglans regia were grown from a 15 milligram size to young plants and planted in soil. The optimum medium, thus far, was a SKW medium with added glutamine, although other media were also useful. The growth of these embryonic axes was much less than embryos with their cotyledons or part of their cotyledons attached. The known constituents of the cotyledons, such as the amino acids, need to be tested for their effect on growth. The objective is to devise an optimum medium for embryonic growth into young plants.

c) Embryo-like structures (embryoids) were induced to form from the cotyledons of the walnut cultivars Amigo, Hartley, Payne, Scharsch-Franquette, and J. hindsii. The best medium for this induction is a low auxin (IBA) high cytokinin (benzyl-aminopurine) medium with the amino acid, glutamine added. The embryoids often resemble embryos with small cotyledons, but may also take other forms. The embryoids grow on the exposed surface of the cotyledon and in many cases formed other embryoids when placed on suitable nutrient media. This is referred to in the literature as repetitive embryogenesis (1) and may be useful as this system of propagation is developed.

2. Endosperm

The endosperm cultures of all the walnut cultivars produced neither callus nor any sign of regeneration. The endosperm was available for only part of its developmental stage in the fruit (4), so it is possible that the appropriate stage was missed or the correct medium was not provided. There are good reasons to pursue this in the spring when earlier stages of the endosperm will be available and an array of essential nutrients can be tested. Plants derived from endosperm would have a unique genetic base which would be useful for rootstock selection related to disease resistance.
3. Protoplasts

Protoplasts were prepared from J. hindsii callus cultures and similar techniques gave some protoplasts from Serr leaves. These procedures need to be refined and carried to the stage of callus formation, followed by the regeneration of plants, either through organogenesis (shoot and root formation) or by somatic embryogenesis (via embryoids).

CONCLUSIONS

The program to develop a reliable system for the regeneration of walnut plants from embryos, endosperm or protoplasts has shown progress in the last six months, especially in the area of somatic embryogenesis with the formation of embryo-like structures from walnut cotyledons. Emphasis will be placed on this area of research during the next year. The objective will be to improve the system for inducing embryoids from walnut leaves and growing these into plants. Concurrently, and in the following year we expect to continue some of the work on endosperm and protoplasts. Plants derived from these methods would be subjected to screening procedures for specific disease resistance, such as Phytophthora and Blackline.

REFERENCES


