IN VITRO REGENERATION OF LENTIL (*Lens culinaris*); PROTOCOLS AND CHALLENGES

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ABSTRACT
Grain legumes (Fabaceae) have a lot of uses in human nutrition owing to their protein-rich seeds. To enhance grain legume production and yield per unit area, new varieties are needed to developed and spread. Under such conditions, grain legumes have a significant potential to adopt biotechnological approaches for improving grain legumes, such as tissue culture. Fabaceae species are difficult to regenerate in vitro, are mainly recalcitrant and frequently exhibit high genotypic specificity. Grain legumes have lower regeneration potential than forage legumes. Plant regeneration from somatic cell in vitro culture has proven challenging in large seeded leguminous species, as opposed to forage legumes. Morphogenesis is exceedingly slow in them, and associated concerns such as the production of albinos, vitreous tissues, and no-response in dedifferentiated calli pose major challenges to the employment of in vitro techniques in legume improvement. Within those species, the lentil was widely studied in tissue culture experiments to conduct an efficient propagation and breeding methods. There has a significant potential to adopt biotechnological technologies for improving and lentils, such as tissue culture. However, due to its recalcitrant disposition in in vitro environment, cultivar improving through biotechnology approaches appears to be limited and difficult to regenerate. Numerous prior research have been conducted in order to construct successful tissue regeneration systems in vitro conditions and to investigate the possibilities of adopting biotechnology to lentil development initiatives. In this study, in vitro regeneration protocols and difficulties of lentil plant have been investigated.
Keywords: In Vitro Optimization, *Lens culinaris*, Tissue Culture

MERCİMEĞİN (*Lens culinaris*) İN VİTRO REJENERASYONU; PROTOKOLLER VE ZORLUKLAR

ÖZET
Yemeklik tane baklagiller (Fabaceae), protein açısından zengin tohumları nedeniyle insan beslenmesinde yaygın olarak kullanılmaktadır. Yemeklik tane baklagil üretiminin ve birim alandan elde edilen verimin arttırılması için yeni çeşitlerin geliştirilmesi ve yaygınlaştırılması gerekmektedir. Bu koşullar altında yemeklik tane baklagillerin, doku kültürü gibi türlerin iyileştirilmesine yönelik biyoteknolojik yöntemleri kabul etme konusunda önemli bir potansiyeli vardır. Fabaceae türlerinin in vitro rejenerasyonu zor olmakla birlikte in vitro ortamda çoğunlukla dirençlidir ve sıklıkla yüksek genotipik özgüllük sergiler. Yemeklik tane baklagillerin in vitro rejenerasyon potansiyeli yem baklagillerine göre daha düşüktür. Yemlik baklagillerin aksine, in vitro somatik hücre kültüründen bitki rejenerasyonunun yemeklik tane baklagiller türlerinde zor olduğu kanıtlanmıştır.

Morfogenez son derece yavaş olmakla birlikte ve albinoluk, vitreus dokuları ve farklılaşmamış kalluslarda rejenerasyon olmaması gibi ilgili sorunlar, baklagillerin in vitro ortamda rejenerasyon ve geliştirilmesinde büyük zorluklar oluşturmaktadır. Bu türler arasında mercimek etkili bir çoğaltma ve yetiştirme yöntemleri yürütmek amacıyla doku kültürü çalşmalarında geniş çapta incelenmiştir. Mercimek ıslahında doku kültürü gibi biyoteknolojik teknolojilerin kullanılmasında büyük bir potansiyel bulunmaktadır. Ancak in vitro ortamdaki dirençli yapısından dolayı, biyoteknolojik yaklaşımlarla çeşitlerin geliştirilmesi sınırlı ve yeniden üretilmesi zor görünmektedir. Başarılı in vitro doku kültürü rejenerasyon sistemleri oluşturmak ve in vitro mercimek rejenerasyonunu geliştirme girişimleri, biyoteknolojiyi uyarlama olanaklarını araştırmak için çok sayıda araştırma yapılmıştır. Bu çalışmada mercimek bitkisinde in vitro rejenerasyonda kullanılan protokoller ve rejenerasyon aşamasındaki zorluklar araştırılmıştır.
Anahtar Kelimeler: Doku Kültürü, *Lens culinaris*, In Vitro Optimizasyon

INTRODUCTION

Grain legumes, often known as pulses, are an important agricultural crop across the world. Pulses are a traditional food crop grown in Latin America, Asia and Africa. Due to widespread protein deficit and a severe scarcity of animal proteins, grain legumes are becoming more and more important in many underdeveloped countries. (Bressani 1973). Moreover, grain legumes are thought to be the main source of protein for inland fish production and animal feed. Also, grain legumes have an unrivaled capacity to fix nitrogen symbiotically, boosting soil fertility overall. (Sarker et al., 2012).

Lentils have a long culinary history in the Old World and are a major crop throughout the Mediterranean and the Near East, where they originated (Ladizinky, 1979). In human nutrition, they are an important source of protein and fiber. Lentils are also valued as a rotation crop for replenishing soil nitrogen levels, and their drought and cold tolerance make them well-suited to low-input farming systems (Ocampo et al., 2000). Poorer yields have been cited on a variety of factors, including insect and disease susceptibility, massive bloom drop, and post-harvest losses. (Solh, 1984).

To enhance the yield and production of lentils per unit area, new varieties must be developed and distributed. Numerous efforts have been conducted to develop lentil varieties through conventional breeding techniques, but these efforts have failed due to a narrow genetic base and a lack of available and feasible germplasm for lentil improvement. There is tremendous possibility for embracing biotechnological techniques to lentil enhancement. Actually, several research have been conducted in previous years to establish acceptable in vitro regeneration systems and to evaluate the viability of using biotechnology into lentil improvement projects. Under such conditions, lentil has a significant potential to adopt biotechnological technologies for improving lentils, such as tissue culture (Küplemez and Yıldırım, 2020).

The availability of totipotent tissues that rapidly respond to in vitro methods is required for the effectiveness of tissue culture-based techniques in legumes. However, because to its recalcitrant nature, narrow genetic base and high genotypic specificity, lentil improvement via biotechnology technologies appears to be limited and difficult to regenerate. As with other grain legumes, there are limiting factors in in vitro studies such as extremely slow morphogenesis during the in vitro regeneration phase of these plants, problems such as albinism, lack of regeneration of vitreous tissues and undifferentiated calli occur (Khanam et al.,1995; Polanco and Ruiz, 1997; Khawar and Ozcan 2002; Sarker et al., 2003; Khawar et al., 2004; Sevimay et al., 2005; Pratap et al., 2010). In this manuscript, protocols and challenges of in vitro regeneration of lentil will be represented between the years of 1979 and 2020.

RESULTS AND DISCUSSION

Literature from Google Scholar between the years of 1979-2020 were referenced in this research in scope of in vitro studies. First shoot regeneration by organogenesis or somatic embryogenesis has been reported by Bajaj and Dhanju (1979), Williams and McHughen (1986), and Saxena and King (1987). The researchers suggests that using cytokinin in the medium a keystone for lentil shoot regeneration.

In Vitro Protocols of Lentil

A number of research have been studied in lentil to conduct whole plant renegeration systems and being a basis for future biotechnology research. In most studies, researchers focused on cytokinin/auxin interaction, rooting and different explants and cultivars.

Williams and McHughen (1986) reported that shoot regeneration occurred in the medium containing only 10mg/L kinetin and 0.1mg/L GA_3. Regenerated shoots were formed only in calli and epicotyl obtained from shoot meristems. Calli obtained from cotyledons did not show any development.

Saxena and King (1987) conducted research on morphogenesis from callus cultures in lentil plants. Somatic embryogenesis was performed from callus cultures obtained from embryos. MS and B5 media were used. Calli in B5 medium did not show much growth in control, BA and IBA medium.

Polanco and Ruiz (1997) tested the effect of 6-benzylaminopruine (BAP) on rooting development in lentil plants in vitro and in vivo. Shoot tip, first nodes, side branch shoot tip and immature seeds were cultured. Plants applied 2.25 and 0.225 mg/L BAP showed an increase in multiple side branches. The resulting shoots (between 4.6% and 39.9%) were placed in rooting medium containing 2 mg/L indoleacetic acid (IAA). As a result of in vivo applications, it was observed that BAP application caused a decrease in root development and root mitotic index. Also Polanco and Ruiz (2001) cultured immature seeds of 4 lentil cultivars in 10 different environments to determine their effect on plant regeneration. Shoot regeneration was observed in almost all media containing kinetin (KIN), benzylaminopurine (BAP) and indole acetic acid (IAA). The best rooting was obtained in Verdina cultivar with 1 µM naphthalene acetic acid (NAA).

Fratini and Ruiz (2002) investigated the effects of different cytokinins 6-benzyladenine (BA), Kinetin (KIN), Thidiazuron (TDZ) and Zeatin (ZEA) on morphogenesis in lentil (*L. culinaris*) plant in in vitro conditions. Mature lentil seeds. showed good results at all cytokinin concentrations.TDZ and BA application promoted more shoot development than KIN and ZEA application.

Khawar and Özcan (2002) reported that TDZ concentration had a positive effect on multiple shoot regenerations in callus-based cotyledon nodes. 1.25 µM IBA concentration was used for rooting, but rooting was not successful in most of the plantlets. To overcome this problem, micrograft was performed within 3-4 days from seven prominent varieties. As a result of this method, Kayı 91 variety was rooted among seven varieties. After germination, the plants were grown in MS medium for 10 days and were taken to root in IBA medium at 0.25, 0.5, 1.0 and 2.0 mg/L concentrations. In the results obtained after 4 weeks, the highest rooting was observed in the medium containing 0.25 mg/L IBA.

Omran et al. (2008) established shoot regeneration and rooting experiments in lentil plants at different explant and BAP concentrations. Shoots obtained from 2 mg/L BAP medium showed higher rooting than shoots obtained from 3 mg/L BAP and 4 mg/L BAP medium. These results show the feature of BAP concentrations that prevents rooting.

Bagheri et al. (2012) studied the indirect in vitro regeneration capacity of lentil plants on 13 different doses of plant growth regulators and 4 different explants. The best callus was obtained from the medium of 1 mg/L naphthalene acetic acid (NAA) and 1 mg/L zeatin.

During the regeneration phase, the plant growth regulator group containing 2,4-D alone and in combination with other plant growth regulators did not show any shoot or rooting. The highest shoot and rooting rate was obtained from embryos decapitated with quarter cotyledons in 1 mg/L naphthalene acetic acid (NAA) and 1 mg/L zeatin medium during callus induction.
Özdemir and Türker (2014) conducted a study on shoot regeneration and rooting in shoot tip, stem, hypocotyl, cotyledon and root explants, including 16 different cultivars. While the medium containing 4 mg/L BAP produced the best shoot regeneration in shoot tip explants obtained from Green 21 cultivar, the best shoot regeneration was obtained in 1 mg/L BAP medium in cotyledon explants taken from the Özbek cultivar. Additionally, shoots treated with IBA were rooted more easily in 1.87 mg/L NAA medium.
Küplemez and Yıldırım (2020), conducted a study to determine effect of cytokinin and auxins on vascular tissues of lentil cultivar of Çiftçi. Intact lentil seedlings treated with different concentrations of BAP (1 mg/L) and BAP (1mg/L)+NAA (0.3, 0.6, 0.9, 1.2 mg/L) concentrations with periodic intervals of 7, 14, 21, 28, and 35 days under in vitro conditions. The best results were often shown under seven-day culture conditions for medium. Extensive days of culture treated with high concentrations of BAP and NAA resulted in damaging effect on vascular tissues and plant development. The xylem region was observed to have the largest thickness when 1 mg/L BAP treatment was applied alone, whereas the phloem region had the highest thickness in the control group. Furthermore, deformation and shrinkage in the phloem and xylem regions were impacted on by large doses of BAP and NAA, which also caused the stem radius to shrink. The damaging effects of BAP and BAP combined with NAA on the vascular tissues inevitably affected the plant's roots system and pericycle cells.

Challenges

These findings show that one of the primary issues with lentil in vitro response is limited rooting efficiency. The use of cytokinins, either alone or in conjunction with auxins, has been a typical aspect of the protocols used to accomplish adventitious shoot regeneration in culture media. Polanco et al. (1988) demonstrated that benzyladenine (BA) was the most successful in stimulating shoot production from calli, but researchers also noted that BA inhibited root growth.
Rooting process from in vitro-regenerated shoots appears to be the key issue for developing full plant regeneration systems in lentil, since roots were formed only sporadically, if at all, from regenerated shoots in all of these investigations. Williams and McHughen (1986) achieved a rooting effectiveness of 11% only when shoots were transplanted to sand. Polanco et al. (1988) proposed that BAP may suppress in vitro root development in lentil. According to
It is evident from these studies that BAP or BA treatments in vitro medium inhibited root formation (Polanco and Ruiz, 1997; Omran et al., 2008; Küplemez and Yıldırım, 2020).
Also, according to Khawar and Özcan (2002), TDZ concentration has a beneficial effect on numerous shoot regenerations in callus-based cotyledon nodes. The concentration of 1.25 M IBA was used for rooting, although most of the plantlets were unable to forming roots. To address this issue, seven prominent cultivars were micrografted within 3-4 days. Kay 91 was rooted among seven cultivars as a result of this strategy.
A study conducted by Polanco and Ruiz (1997), revealed that in vitro and in vivo benzylaminopurine (BAP) inhibited root formation on both environments and caused drastic reduction of the mitotic index of the root meristem.
In our former study, we observed that 1 mg/L BAP treatment alone or combined with 0.3 mg/L and 0.6 mg/L NAA shrinked phloem width while 0.9 mg/L and 1.2 mg/L NAA combined with 1 mg/L BAP treatment found to be more wider phloem width. Also prolonged 1 mg/L BAP treatment alone caused more damage on phloem which thought to be had a damaging effect on pericycle cells and rooting of the plant.

Furthermore, BAP and BAP combined with NAA treatments and exposure demonstrated a clear influence on rooting with 0.5 mg/L IBA. Only control group shoots precultured for 21, 28, and 35 days rooted. On shoots precultured on MS media for 7 and 14 days, no roots was observed. Any, BAP, or BAP combined with NAA pretreatment decreased root induction. It was discovered that BAP and NAA-treated shoots inhibited rooting mostly due to damaging and shrinking of vascular bundles in the root zone (Küplemez and Yıldırım, 2020).

CONCLUSION

In conclusion, while BAP or BA is usually added to tissue culture medium to stimulate shoot regeneration, the concentration of BAP/BA and culture time in media must be carefully determined in the lentil micropropagation process to minimize BAP/BA-induced inhibition of roots in regenerated shoots. Also utilizing cytokinins in culture media such as BAP, TDZ or KIN could effect the rooting rates. In vitro studies on lentil is still few. In future research, determining how to reverse these negative effects on plants with different plant growth regulators and culture times is important for in vitro culture of lentil plants.

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