Review Article

Some Important Factors Influencing Tissue Culture Response in Wheat

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Abstract | Various methods and techniques have been developed and tested to improve important cereal crop species since quite long time. Among the prominent techniques, traditional plant breeding has been on leading edge. The discovery of molecular genetics and biotechnological techniques, such as genome mapping, tissue culture, genetic transformation and *in vitro* regeneration offered new dimensions of research to improve important crop species including wheat. The tissue culture by itself has increased the crop improvement potential to develop new cultivars with desirable traits through somaclonal and gametoclonal selection. While successful tissue culture is critical for genetic transformation, it has been facing greater impediments in wheat and related crop species. Its success depends largely upon the average effect of several important factors including explant tissue, culture medium, plant genotypes, and their interactions, the detail of which is not fully explored. Current article described this issue in detail. The information synthesized would be important to select and manipulate appropriate factor(s) for successful wheat tissue culture and regeneration that in turn would help its improvement.

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Introduction

A number of conventional and modern plant breeding methods and techniques have been developed and practiced in the last several years to improve wheat crop for the human society. These techniques have greater limitations because of the technical implications, longer time requirements and most importantly narrow gene pool in wheat (Vasil, 2007; Godfray et al., 2010; Gao et al., 2013), thus warrant discovering and following better alternatives.

The development and applications of modern genetic transformation tools offered greater potential to improve wheat for better yield and quality by breaking the barriers among interspecies and even inetergeneric transferring of the novel genes of improved characteristics. However, plant transformation requires an efficient, reliable, stable and reproducible tissue culture system with optimized protocols in the first place (Yu et al., 2008; Noor et al., 2009). Being the most important food crop, wheat has been greatly experimented for genetic transformation but was found to be recalcitrant towards *in vitro* regeneration repeatedly (Ganeshan et al., 2006). For better understanding of tissue culturing in wheat, information on the fac-

tors controlling both callus induction and regeneration is important. Several factors have been reported to affect tissue culture responses of wheat, specifically formation of embryogenic calli and plant regeneration (Mitić et al., 2006). These factors include explant tissue (Vasil, 1994), growth media (Mathias and Simpson, 1986), and plant genotypes (Hess and Carman, 1998). To date, several studies have been conducted describing factors affecting the plant regeneration ability from wheat immature embryos (Ben Amer et al., 1995, 1997). As a part of long-term project on wheat biotechnology, we have evaluated information regarding important factors that influence the wheat tissue culture. The information in the current article would not only be important to understand the wheat *in vitro* regeneration but will also help to select appropriate factor(s) for its long term improvement through tissue culture and genetic transformation.

Factors Affecting the Tissue Culture Response in Wheat

Explant tissue

Various explants' tissues have been reported for wheat callus induction and regeneration including shoot tips (Viertel and Hess, 1996), glumella and lemma (Lu, 1992), inflorescence stem sections (Benkırane et al., 2000), isolated anthers (Konieczny et al., 2003), nodes (Lu, 1988), coleoptiles (Benkırane et al., 2000), shoot apical meristems (Haliloglu et al., 2006), leaf base (Wang and Wei, 2004), and mature and immature embryos (Ozgen et al., 1996; Sarker et al., 2007).

Majority of the reports showed that the type of explants greatly influence the frequencies of callus induction and regeneration in wheat tissue culture (Tamas, 2004; Patel et al., 2004; Shariatpanahi et al., 2006; Redha and Talaat, 2008) along with genotype (Fennell et al., 1996; Filippov et al., 2006; Mahmood et al., 2012) and media composition (Przetakiewicz et al., 2003; Tamas et al., 2004; Mahmood et al., 2012). Every explant differs in its response to callus induction and regeneration (Delporte et al., 2001). In both callus induction and regeneration immature and mature embryos and immature inflorescence have been reported as the most responsive and frequently used explants (Li et al., 2003; Shah et al., 2003; Haliloglu et al., 2005) for tissue culture (Table 1).

Immature embryos: Immature embryos have been the most effective explants used for regeneration studies (Table 1). For the very first time Shimda (1978) used these as an explant in wheat and successfully obtained the regenerated plants. Since then, many researchers have used immature embryos for wheat transformation because of their potential to generate embryogenic callus, and high regeneration capacity of large number of genetically fertile transformed plants (Arzani et al., 1999; Ozgen et al., 1998; Jones, 2005; Chauhan et al., 2007). Many studies on wheat regeneration have proved that both callus induction and regeneration ability of immature embryos is far better than any other explant in the family Gramineae (Lü et al., 2007).

However, while using the immature embryos as explants, optimization of several factors including embryo age, media composition and especially the auxin concentration play pivotal role in callogenesis and regeneration. The most important of all is the age of the immature embryos; it is observed that immature embryos older than twenty seven days were found to fail in regenerating the whole plant. The explants younger than a specific size also could not produce the desired outcomes. Therefore, a specific developmental stage is always required (i.e. 0.8–1.5 mm in diameter) to collect the immature embryos for good results (Wu et al., 2009). It is observed that when the source plants are under any biotic or abiotic stresses, it lowers the regeneration ability of the immature embryos (Carman et al., 1988; Dodig et al., 2008; Mitic et al., 2009).

Though immature embryos are reported to be the appropriate source for calli induction and regeneration

Table 1: *Most frequently used explants for callus induction and regeneration*

S.No	Explant	References
1.	Immature Embryos	Shimda, 1978; Arzani and Mirodjagh, 1999; Sarker and Biswas, 2002; Pellegrineschi et al., 2004; Tang et al., 2006; Lü et al., 2007; Mahmood et al., 2012; Khaled et al., 2013.
2.	Mature Embryos	Delporte et al., 2001; Noor et al., 2009; Afzal et al., 2010; Jiang-ping et al., 2010; Abdallah et al., 2012; Aydin et al., 2012., Rashid et al., 2012; Hakam et al., 2015.
3.	Leaf and young inflorescence	Ozias-Akins and Vasil, 1982; Redway et al., 1990; Casas et al., 1997; Benkırane et al., 2000; Chugha and Khurana, 2003; Wang and Wei, 2004; Yu et al., 2012.

(Repellin et al., 2001; Li et al., 2003; Haliloglu et al., 2005; Jones, 2005; Chauhan et al., 2007), their use has also limitations in influencing environmental conditions on the donor plants (Carman et al.,1988). Immature embryos also pose a difficulty in demanding the extra labour and maintenance of the donor plants. Moreover use of immature embryos for efficient callus induction and regeneration response depends on the most suitable growth stage of wheat (Repellin et al., 2001) and sophisticated growth chamber facilities. On the other hand, mature seeds of wheat are readily available throughout the year, hence can be used for plant regeneration in any convenient time (Rahman et al., 2008).

Mature embryos: Mature embryo is another most frequently used explant for the tissue culture in wheat. Their use as explant was first introduced by the Zhou and Lee (1983) in wheat with successful calli induction and plantlet regeneration. Since then, mature embryos have been exploited as explants with varying degrees of success (Özgen et al., 1998; Delporte et al., 2001; Sharma et al., 2005). Mature embryos used as explants showed high percentage of regeneration response (Ozgen et al., 1996) and they also pose an ease in being available around the year (Ding et al., 2009). In addition, minimal variability is observed in the plants regenerated through the mature embryos because of the physiological state (Yu et al., 2008). However, researchers have used different ways in treating the mature embryos for tissue culture like endosperm-supported embryo (Ozgen et al., 1998; Filippov et al., 2006), thin mature embryo fragments (Delporte et al., 2001; Mendoza and Kaeppler, 2002) longitudinally bisected mature embryos (Yu et al., 2008), mesocotyl and epicotyle of mature embryo and pretreatment of embryo with high levels of auxin such as 2, 4-D prior to or during culture. Some researchers utilized the callus derived from mature embryos to observe the function of genes that govern the processes of transportation, response, induction, synthesis and degeneration of auxin (Chen et al., 2009).

Though many researchers have replaced the immature embryos with the mature ones (Özgen et al., 1998; Delporte et al., 2001; Sharma et al., 2005), still callus rate derived from mature embryos is lower than that from the immature ones. Therefore immature embryos are still a better choice for wheat regeneration studies. To overcome the limitations posed by the use of immature embryos, more frequent experiments on relationships between genotype and the growing season is of great demand.

Leaf and immature inflorescences: Leaf is another suitable source of callus induction and regeneration in wheat. Leaf has been a preferable explant being devoid of meristematic cells except being present only at the base. Leaf basal segments of seedlings frequently consisted of mixed tissues including leaf and coleoptiles tissue (Chugh and Khurana, 2003), the callus derived from such explant is totally undifferentiated and largely utilized in the molecular investigations of plants (Ahuja et al., 1982)

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Immature inflorescence is also a preferred choice of explant as the compact and nodular callus formation has been observed from such tissues (Ozias-Akins and Vasil, 1982). It is also important to note that immature inflorescence not only produced good quality callus in wheat but also had a pronounced effect on callus initiation and somatic embryogenesis in allied species of *Triticeae* including Rye, Barley and Triticale (Marcinska et al., 1995; Barro et al., 1999). Moreover, calli produced by immature inflorescence is more morphogenic in comparisons with other explants used because they contain abundance of suppressed meristematic regions that become active when come in contact with the nutrient medium (Maheshwari et al., 1995). Karesa et al. (2004) compared callus induction and regeneration in different Croatian wheat cultivars using inflorescence as explant and reported the high percentage of regeneration and callus induction with the addition of Picloram in the medium. Almost 100% regeneration was achieved in the winter wheat with the inflorescences as explants (Kavas, 2008).

Though immature inflorescence and young leaves are the preferred choice of calli induction and regeneration in wheat, their use is limited because of the specific growth period and developmental stages.

Genotype

Plant genotype is of prime importance and is well documented to affect callus induction and plant regeneration in all major cereal species (Ozgen et al., 1998). Role of genotype in wheat tissue culture remained an important subject in several studies (Tyankova et al., 2006). It was determined that wheat tissue culture response is polygenic in nature (Bregitzer and Campbell, 2001) and can be transmitted by heredity (Chevrier et al., 1990). However, there is an inadequate under-

standing of genetic control of callus induction and regeneration. In order to overcome this limitation many investigations have been carried out in wheat to find out the chromosomes that control the tissue culture response (Tyankova and Zagorska, 2001). Mendelian inheritance, quantitative genetics and translocation line analysis remained some of the investigation tools used for studying the chromosomal effects on wheat tissue culturing. In addition valuable experimental stocks like aneupoloids, translocation lines and chromosomal substitution lines were also used for studying the wheat tissue culture response (Mathias and Fukui, 1986; Higgins and Mathias 1987; Ben Amer et al., 1997).

Many studies concentrated on the evaluation of single chromosomes of wheat genome to find its effect on tissue culture (Felsenburg et al., 1987). Using substitution lines of Chinese Spring and Cappelle Desprez, Higgins and Mathias (1987) reported the effect of chromosome 4B on wheat callus induction and regeneration. While from A genome of wheat, chromosomes 1A (Ben Amer et al., 1997) and 2A (Tyankova et al., 2006) were found governing the callus induction and regeneration response. Control of chromosome 1D and 4D on wheat tissue culture was reported by Tyankova et al. (2006).

These results indicate that in vitro response in wheat though controlled by the specific chromosomes, there is great contradiction regarding genetic control of tissue culture response. It is very clear from such findings that the biological processes behind the in vitro wheat response are quite complex and need to be further explored.

Media composition-Chemical environment

Though callus induction is a wound healing response in nature, it can be initiated *in vitro* on a contact of cut surface or exposed part of the tissue with the nutrient medium or cell that can be proliferated with the addition of growth regulators (Mc Clintock, 1984). The tissue culture frequency is greatly influenced by the medium composition (Przetakiewicz et al., 2003; Tamas et al., 2004). Generally, for wheat tissue culture, the basic salt formulation used in solidified MS (Murashige and Skoog, 1962) medium supplemented with vitamins, hormones and sugars. All the components of media have a pronounced effect on the tissue culture response in wheat. Major components that influence the callus induction and regeneration are listed in Table 2.

Sugar: Sugar is the source of carbon in the tissue culture medium, that greatly influence the somatic embryogenesis and efficient plant regeneration. Sucrose and maltose are the most often used carbon sources; however in comparison, maltose is reported to be more significant that greatly enhance the callus formation (Last and Brettell, 1990; Gadaleta et al., 2006). It is believed that slow hydrolysis of maltose to glucose and relatively high and constant osmolarity in the medium enhance the production of effective calli (Mendoza and Kaeppler, 2002). One of the most important factors that controls and regulates the tissue culturing is the use of plant growth hormones or their synthetic analogues.

Plant growth hormones: Most commonly used plant growth hormones are auxins, cytokinins, gibberellins, absisic acid and ethylene. Among frequently used plant growth regulators are the auxins and cytokinins (Table 2) (Pullman et al., 2005).

Table 2: *Commonly used auxins and cytokinins in wheat callus induction and regeneration*

S.No	Cytokinins	Auxins
1.	Benzylaminopurine (BAP)	2,4 Dichlorophenoxyacetic acid (2, 4-D)
2.	Absisic acid (ABA)	2-Methoxy-3,6-dichlorobenzoic acid (Dicamba)
3.	Zeatin	4-amino-2,5,6-Trichloropicolinic acid (Picloram)
4.	Kinetin (Kn)	2,4,5-Trichlorophenoxyacetic acid (2, 4, 5-T)
5.		Indole-3-butyric acid (IBA)
6.		Indole-3-acetic acid (IAA)
7.		1-naphthylacetic acid (NAA)

Auxins play a vital role in promoting cell division and growth while cytokinins are responsible for enhancing cell division. The most commonly used auxin is 2,4-Dichlorophenoxyacetic acid (2,4-D) but some other auxins including 2,4-5-trichlorophenoxyacetic acid (2,4,5-T) and Dicamba are also in frequent use in tissue of wheat with varying degree of success (Slater et al., 2008). The most frequently used cytokinins are the zeatin and benzylaminopurine (BAP). Absisic acid (ABA) is responsible for preventing the cell division; therefore, it is most commonly used to induce the distinct developmental pathways like somatic embryogenesis. Plant hormones show great variations in

responses to species and cultivars. However, the ratio of auxin to cytokinins pose a great effect in plant developmental processes, as a high auxin to cytokinins ratio promotes shoot formation while root growth is promoted when cytokinins ratio is higher to auxin ratio (Slater et al., 2008).

Among auxins, 2,4-D is well known to induce the shoot formation and somatic embryo differentiation (Nbaors et al., 1983; Mahalakshmi et al., 2003). In order to induce an effective callus, an optimum concentration of 2, 4-D is very important.

Higher concentrations of 2, 4-D are reported to produce the chromosomal instability leading to somaclonal variation (Mendoza and Kaeppler, 2002; Satyavathi et al., 2004). Whereas a much lower 2, 4-D concentration decreases the embryogenic ability of the culture (Vasil and Vasil, 1982), an optimum concentration of 2, 4-D enhance the production of somatic embryogenesis (Ozias-Akins and Vasil, 1982; Viertel and Hess, 1996). Yellow compact embryogenic calli formation was reported by various researchers with 2 mg/L of 2, 4-D (Abdullah et al., 2002; Yu et al., 2008). A continuous auxin supply results in the retardation of embryo differentiation, progression and development (Mahalakshmi et al., 2003). However, the genotype and auxin interactions play a great role in determining the callus induction response in wheat. Different genotypes respond in a different manner on the same auxin concentration. It is well known that the effect of auxin vary greatly with the genotype used (Rashid et al., 2009).

Studies indicated that the use of Dicamba has surpassed the efficiency of 2,4-D in many cereals (Trifonova et al., 2001; Przetakiewicz et al., 2003). In addition, its use led to the initiation of larger calli with increased shoots (Mendoza and Kaeppler, 2002). However, it has also good effect on wheat callus induction (Satyavathi et al., 2004) with different concentration from as low as 2 mg/L to 12 mg/L resulting in the embryogenic calli formation and good regeneration rate (Mendoza and Kaeppler, 2002; Kilinc, 2004).

The use of 2, 4-D in combination with Dicamba showed significant effect on the differentiation frequency of callus tissue (Qin et al., 2013). This is based on the fact that when these two auxin were used in combination, the formation of embryogenic callus formation was achieved to 90% regeneration (Nbaors et al., 1983; Qin et al., 2013). It is also reported that 2,4-D and Dicamba induce the embryogenesis (Barro et al., 1998; Campbell et al., 2000) either alone or when used in lower concentrations with cytokinins (Barro et al., 1998; Campbell et al., 2000).

Embryogenic vs Non-embryogenic Callus

It is of great importance to understand as which type of callus is produced from the explant, genotype, and culture media used. The embryonic callus has greater regeneration capability either through embryogenesis or organogenesis. In contrast non-embryonic callus has the ability of proliferation, but cannot regenerate. Embryogenic callus can be visually judged by its specific color from white to pale yellow being compact structure, nodular and globular, in shape and relatively dry in appearance. While non-embryogenic callus can be recognized by yellow or brownish in color, loose textured, irregular in shape and watery in appearance (Mahmood et al., 2012; Hassan et al., 2009).

The distinction of embryogenic calli from the non embryogenic at the time of regeneration stage is generally the least discussed factor (Redway et al., 1990; Delporte et al., 2001; Mendoza and Kaeppler, 2002) in plant tissue culture. Different portions of the embryo or any explant have different capacity of callus formation. Based on the usefulness, only embryogenic callus should be separated from rest of the mass during sub culturing for regeneration (Mac Kinnon et al., 1987). The differentiation between embryogenic and non embryogenic calli is usually done on the basis of their morphology and color (Redway et al., 1990). Precautions need to be followed as this separation largely depends on the callus age, auxin type and genotype used. Older callus are difficult to be recognized for their embryogenicity while eech genotype has different color. Moreover, the auxin used for callus induction also affects the callus color, but separation of embryogenic callus from non embryogenic is very important while transferring to the regeneration medium.

In order to induce the somatic embryogenesis and effective regeneration system in wheat, the combinational effect of both auxin and cytokinins ratio is of vital importance. For obtaining higher regeneration percentage, higher levels of cytokinins are employed either with or without lower auxin levels (Hassan

et al., 2009). Somatic embryogenesis is greatly influenced by the type as well as concentration of the auxins and cytokinins present in the medium. The continuous supply of auxin in the regeneration medium poses a negative effect because they form the gene products that synthesize the mRNA which can inhibit the somatic program. However, lowering the auxin concentration from the medium or complete elimination with the addition of cytokinins greatly enhance the process of embryogenesis (Bregitzer, 1992; Delporte et al., 2001). Most commonly used cytokinins for wheat regeneration are the zeatin, kinetin, benzylaminopurine and absisic acid. Different combinations of cytokinins and auxins have been tested with varying results. Indole acetic acid when combined with Benzyl Amino Purine gave the regeneration response in wheat as almost 40 % (Rashid et al., 2002; Afzal et al., 2010). Whereas, regeneration medium supplemented with zeatin showed 100% regeneration (He and Lazzeri, 2001; Rahman et al., 2008). However there are many other combinations of cytokinin under different concentrations that have been utilized for the efficient regeneration response including IAA with kinetin. Shah et al. (2003) in their experiments obtained highest regeneration percentage of wheat on MS medium containing 4 mg/L. It is generally accepted that a lower auxin and higher cytokinins ratio enhance an efficient regeneration system in wheat.

Conclusions

Wheat improvement through genetic transformation relies largely upon *in vitro* regeneration which in turn requires successful, reliable and stable tissue culture system. Various factors critically influence tissue culture response in wheat, the understanding of which is important to lay successful transformation experiments. Detailed discussion on the available information to these factors was carried out in the current review. This will greatly help devising efficient protocols for wheat tissue culture by selecting most suitable combinations of explant tissue, genotypes and culture media.

Authors' Contribution

Ummara Waheed Khan collected all the information and wrote the first draft of the manuscript. Mohmmad Maroof Shah conceived the idea, synthesized the information and assisted in writing up with multiple revisions. Raza Ahmed and Irum Shahzadi assisted in revisions and corrections. All authors read and approved the final manuscript.

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