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Phenotypic evaluation of scutellum-derived calluses in 'Indica' rice cultivars

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ABSTRACT

By using amenable MS based medium containing 4 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.4 mg l^{-1} benzyl-aminopurine (BAP), 30 g l^{-1} sucrose, 8 g l^{-1} Agar-agar, qualitative and quantitative traits of calluses initiated from four genetically and commercially valuable Northern Iranian rice cultivars including Hashemi, Hasani, Gerdeh, and Gharib were studied. Five seeds were placed in each Petri dish and three replicates of eight Petri dishes per replicate were incubated in a growth chamber at 25 ±2 °C in the dark and the averages for every replicate were employed in the analyses. Several important parameters related to callogenesis of the cultivars including rate of non-viable seeds, necrotic, scutellar, slow growing, and non-scutellar calluses, simultaneous callus induction from scutellar and non-scutellar tissues, seeds with appropriate callus, and root emergence were compared. Accordingly, calli of Gharib and Hashemi were highly responsive in callogenesis, while Gerdeh and Hasani produced dissatisfying calluses. Necrotic calluses, scutellar calli, and non-viable seeds were positively correlated with each other; although they were negatively correlated with non-scutellar calli, simultaneous scutellar and non-scutellar calli induction, and root emergence. The results of the present study are expected to be the first promising step to generate genetically manipulated Iranian indigenous rice cultivars.

Key words: tissue culture, callogenesis, necrosis, *Oryza sativa*, 'Gharib', 'Hashemi', 'Gerdeh', 'Hasani'

IZVLEČEK

FENOTIPSKO VREDNOTENJE IZ SKUTELUMA PRIDOBLJENIH KALUSOV IZBRANIH SORT 'INDICA' RIŽEV

Z uporabo MS medija, ki je vseboval 4 mg l^{-1} 2,4-diklorfenoksi ocetne kisline (2,4-D), 0.4 mg l^{-1} benzil-aminopurina (BAP), 30 g l^{-1} saharoze in 8 g l^{-1} agarja so bili preučevani kvalitativni in kvantitativni znaki kalusov, pridobljeni iz štirih genetsko in komercialno priznanih sort riža ('Hashemi', 'Hasani', 'Gerdeh', and 'Gharib') iz severnega Irana. Po pet semen je bilo položeno v vsako od osem petrijevk v treh ponovitvah, ki so jih inkubirali v rastni komori pri 25 ±2 °C v temi. Povprečje vsake ponovitve je bilo uporabljeno v analizah. Primerjani so bili pomembni parametri kalogeneze kot so: število nekalečih semen, nekrotični, skutelarni, počasi rastoči in neskutelarni kalusi, simultana indukacija kalusov iz skutelarnih in neskutelarnih tkiv, semena s primernim kalusom in izraščanje korenin. V kalogenezi sta bili zelo odzivni sorti 'Gharib' and 'Hashemi', medtem ko sta sorti 'Gerdeh' and 'Hasani'dali neustrezne kaluse. Nekrotični kalusi, skutelarni kalusi in nekaleča semena so bili med seboj v veliki pozitivni korelaciji in v negativni korelaciji z neskutelarnimi kalusi, simultano indukcijo skutelarnih in neskutelarnih kalusom in nastankom korenin. Iz rezultatov te raziskave pričakujemo prvi obetajoči korak v pridobivanju genetsko spremenjenih domačih sort iranskega riža.

Ključne besede: tkivne kulture, kalogeneza, nekroza, *Oryza sativa*, 'Gharib', 'Hashemi', 'Gerdeh', 'Hasani'

1 INTRODUCTION

Rice is the second most widely cultivated cereal in the world, after wheat, and is a staple food for over half the world's population. In recent years, considerable efforts have been directed towards the improvement of important agronomic traits of rice through tissue culture based *Agrobacterium*-mediated transformation techniques. However, Indica subspecies is the most recalcitrant one to

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Agrobacterium-mediated transformation and tissue culture techniques. Furthermore, differences in callus growth were even reported within Indica cultivars (Ge et al., 2006). These between-cultivar differences restrict the application of tissue culture techniques to a few rice cultivars (Lin and Zhang, 2005; Ge et al., 2006).

Healthy and actively growing calli are prerequisite for transformation by *Agrobacterium* (Hiei et al., 1994) and biolistic methods (Cao et al., 1992). Short period of tissue culture minimizes the possibility of somaclonal variation and thus improves the fertility of transgenic plants (Toki et al., 2006). Indeed, it has frequently been the plant tissue culture technology, rather than the transformation process itself, that has been the limiting step in achieving efficient transformation (Herrera-Estrella et al., 2005)

Several highly efficient tissue culture systems for japonica and Indica rices have recently been established (Hiei et al., 1994; Lin and Zhang, 2005; Toki et al., 2006; Hiei and Komari, 2008). MS (Murashige and Skoog, 1962) is a widely used medium in Indica rice tissue culture (Lin and Zhang, 2005; Ge et al., 2006; Yan et al., 2010). In Indica rices originated in Iran, robust and highly applicable techniques for tissue culture have never been established, regarding the lack of extensive researches in the *in vitro* culture of Iranian indigenous rice, particularly Northern Iranian cultivars. The mentioned issues impede rice tissue culture; so transformation in Indica subspecies, especially Iranian rice cultivars, faces difficulties.

Nowadays, Hashemi is the most popular cultivar in Northern Iran, which is cultivated in most rice fields of Guilan province in Iran. To the best of our knowledge, these cultivars have never been used to investigate their culturability. In the unique study reported here, mature embryos of four Northern Iranian indigenous rice cultivars (*Oryza sativa* L.) were used to assess the effect of an amenable MS based medium on the initiated calli quality and quantity.

2 MATERIALS AND METHODS

This experiment was conducted in the Biotechnology Laboratory of the faculty of Agricultural Sciences, The University of Guilan, Rasht, Iran.

2.1 Plant materials and sterilization

Mature seeds of four indigenous rice (*Oryza sativa* L.) cultivars, including Hashemi (Hm), Hasani (Hn), Gerdeh (Gr), and Gharib (Gb), obtained from the Rice Research Institute of Iran (http://berenj.areo.ir), were used. Rice caryopses were manually husked, sterilized for 30 min in sodium hypochlorite 10%, and then soaked in hydrogen peroxide 1% (W/W) for 3 h. They were sterilized again in sodium hypochlorite 10% for 20 min (Ozawa, 2009). After every stage of sterilization, seeds were rinsed in sterile distilled water three times. Seeds were carefully inspected for any malformation, immaturity, and stains, before and after hulling.

2.2 Callus induction and culture

After rinsing, embryos were placed on an induction medium with the scutellum facing upwards. The induction medium was MS basal medium (Murashige and Skoog, 1962) containing 4 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.4 mg l^{-1} N^6-Benzyladenine (BA), 30 g l^{-1} sucrose, 8 g l^{-1} Agar-agar. Medium was adjusted to pH 5.8 before the addition of Agar-agar prior to autoclaving. Five seeds were placed on each Petri dish and sealed with Parafilm. For each cultivar three replicates of eight Petri dishes per replicate were incubated in a growth chamber at 25 ±2 °C in the dark and the averages for every replicate were employed in the analyses.

2.3 Observation and statistical analysis

Appearance and proliferation of calli were surveyed and documented after 3 weeks. By using Microsoft Office Excel package the percentages of non-viable seeds (NVS), necrotic calli (NC), scutellar calli (SC), slow growing calli (SGC), non-scutellar calli (NSC), simultaneous callus induction (SCI) from scutellar and non-scutellar tissue, seeds with appropriate callus (AC) (at least 3mm in diameter is defined as appropriate callus,

here), and root emergence (RE) were evaluated for the four Northern Iranian indigenous rice cultivars.

3 RESULTS AND DISCUSSION

Before conducting this experiment, seeds of the four cultivars had been being kept in refrigerator (4 °C) for one year. Thus, for each of the cultivars, some of the cultured seeds were non-viable. However, Hn had the highest, while Gb had the lowest rate of NVS (Fig. 1). Long-term storage augments NVS, which is different for each cultivar. Loss of vigour and viability through dry storage comprises a wide range of degenerative events that accumulate over time and trigger loss of viability (Smith and Berjak, 1995). Several findings have shown that reactive oxygen species (O^{-}_{2} and H_2O_2), play an important role in seed deterioration during aging (Sung and 1996). However, there are compelling evidences that hydrogen peroxide may act as a signaling molecule in plants mediating some hormone-regulated processes (Kwak et al., 2006; Vranová et al., 2002). Contrary to earlier views, it is suggested that exogenous application of hydrogen peroxide promotes germination, that indicates a positive role of active oxygen species in germination (Ogawa and Iwabuchi, 2001; Sarath et al., 2007). Likely, seeds viability has been deteriorated during dry storage, albeit loss of viability differently intensified for each cultivar. Five months after starting the experiment, in a separate germination test, non-viable seeds were accounted for Hm 5.73%, Gr 10.3%, Hn 39.06%, and Gb 5.73%.

Hn produced 77% of NC, which was the highest one among Hm (22%), Gr (21%), and Gb (18%) (Fig. 1). In rice, callus necrosis was most likely to occur in cultivars that produced ethylene at a high rate. Callus growth of these plants was more strongly inhibited by a controlled gas mixture, which had higher ethylene as compared to necrosis-tolerant cultivars (Adkins et al., 1990). It has been shown that $AgNO_3$, an ethylene action inhibitor, increased type II callus production from immature embryos of maize (Songstad et al., 1991). In some cases, ethylene could increase putrescine levels (Lee and Chu, 1992). Similarly, addition of an inhibitor of putrescine synthesis to the culture also restored the ability to regenerate whereby the problem of loss of regeneration ability seen in rice callus cultures may be overcome (Bajaj and Rajam, 1996). Thus, it seems that callus necrosis of rice is due to ethylene which increases cellular putrescine levels. Several studies have been carried out on rice varieties tissue culture and it has been reported that those varieties could be divided into two groups: browning/poor-growth type and non-browning/good-growth type (Abe, 1992; Ogawa et al., 1999). It seems clear that rice cultivars are different in tissue culture necrosis. It is likely that the four cultivars have been examined here have had dissimilar rates of necrosis in callus culture.

In this experiment, NSCs were mostly originated from roots. Gr showed the highest rate (75%) for NSC, while Hn showed the lowest rate (4.54%) (Fig. 1). NSCs are not suitable materials for breeding purposes, for instance *Agrobacterium*-mediated transformation of rice (Hiei et al., 1994; Hiei et al., 1997). It has been reported that calli initiated from scutella had embryogenic potential, while calli that arose from the radicle tended to be non-embryogenic. It has also been suggested that the calli arose from the swollen radicle had a translucent, soft, moist, mucilaginous, and unorganized appearance. These calli did not have regeneration ability (Ge et al., 2006). Emergence of calli derived from non-scutellar tissue of the explants shows the medium has not been defined accurately (George and Sherrington, 1984). In the case of NSC, Gr showed the highest rate of NSC, two-thirds of the whole seeds, whereas Hn produced the lowest amount of NSC, nearly 5% of the whole seeds. SCs are the most suitable tissues for modern breeding methods, particularly in *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). Gr had the lowest rate of SC (25%), while three other cultivars had 65 ±10% of that for cultured seeds (Fig. 1). Results of several studies clearly Indicate that calli initiated from scutella are excellent materials for rice transformation by *Agrobacterium* (Hiei et al., 1994; Hiei et al., 1997). It was observed that calli induced from scutellum were mainly embryogenic. These calli were dry, compact, light yellowish, and nodular in appearance (Ge et al., 2006). In contrast to Gr, three other cultivars produced reasonable

amounts of scutellum-derived calli, especially Hn and Gb. Although Hn had the highest rate of calli initiation from scutella, at the same time it had the highest rate of SGC too. Callus induction from scutellar tissue sometimes coincides with calli from non-scutellar tissue of the same seed, hence termed SCI. While some specimens from each four cultivars had just calli either from scutellar or non-scutellar sources, others had both types of calli. For both types of calli, which mentioned earlier, Hn had about 5% coincident, whereas the percentage of coincident for Hm, Gr, and Gb were 13, 20.8, and 15.3, respectively (Fig. 1).

Rice genotypes are different regarding callogenesis or callus initiation as well as SGC. In this experiment, an appropriate size for a callus has been defined as 3 mm or higher. SGC is not ideal for *in vitro* tissue manipulation or transformation. In the present experiment, calli induced by Hn showed the highest rate of SGC (54.5%), whereas in Hm, Gr, and Gb the rate was 15.4%, 16.7%, and 3.5%, respectively (Fig. 1). The use of actively growing embryogenic calli is one of the most important factors in efficient transformation of rice (Hiei et al., 1997). A SGC needs more time to succeed in breeding aims, furthermore long-term culture increases risks of somaclonal variations. There are two options for cultivars with SGC: (i) optimizing callus initiation medium in favor of cultivar with SGC, or (ii) choosing highly responsive cultivars to callus initiation medium. Regarding the second option, apparently all of the examined cultivars were quite prolific in callogenesis, except for Hn with more than 50% SGC.

While all specimens had almost stunted shoots, they brought out different patterns of roots. In contrast with many cultured seeds that did not have any visible roots, some seeds had however roots. The highest percentage of roots were produced through Gr (62.5%), while in the case of Hn, it was 4.5 times fewer than that of Gr (Fig. 1). Based on the evidences from the present study, root formation is mostly accompanied by root-derived calli, an unintended outgrowth. Rice cultivars have, however, different sensitivity to exogenous hormones application (Khanna and Raina, 1998), hence diversely display altered growth, such as a stunted shoot or root and enhanced formation of adventitious roots. It has been seen that, germination process was inhibited, especially RE, as 2,4-D concentration increased to 2 mg l^{-1}, meanwhile callus proliferation commenced at scutellum region scutellum region. A similar findings were reported by Al-Khayri et al., 1996. It seems that excessive rhizogenesis may result in root-derived calluses, or be a prerequisite for such calluses. This is supported by present data showing Hn and Gr on the opposite sides, showed a positive correlation between RE and NSC formation.

Gb produced the most appropriate calli, whereas Gr showed the most inappropriate calluses (Fig. 1). As mentioned previously, calli derived from scutellar tissue should have satisfactory growth rate and healthy appearance to be promising. During the present study, seemingly, calli, which grew smaller than 3 mm in diameter (the slow growing ones) were not fruitful, and needed to be avoided. NC, also, are not preferable for breeding purposes, as discussed earlier. For instance, unlike the highest rate of scutellum-derived calli, the lowest amount of AC produced by Hn resulted from the highest rate of NC, and SGC, which spoiled its yield.

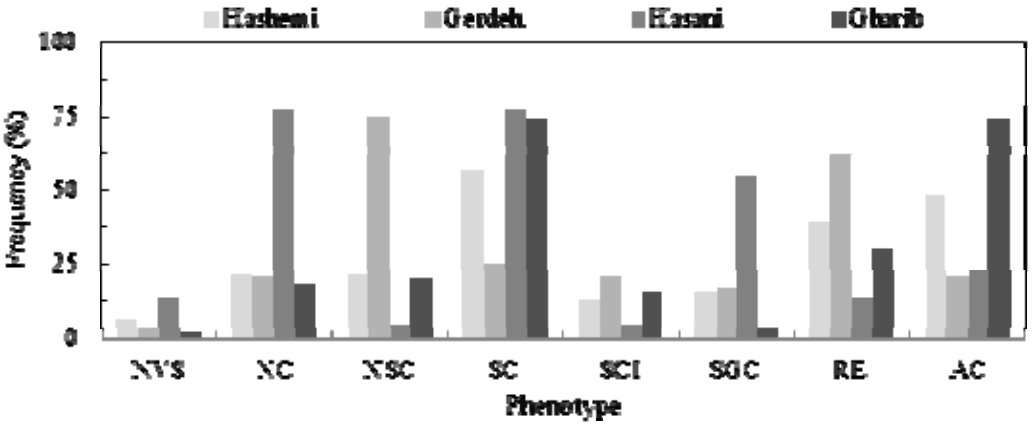

Figure 1: Evaluated rates of: non-viable seeds (NVS), necrotic calli (NC), non-scutellar callus (NSC), scutellar callus (SC), simultaneous callus induction (SCI) from scutellar and non-scutellar tissue, slow growing callus (SGC), root emergence (RE), and seeds with appropriate callus (AC), for the four Northern Iranian indigenous rice cultivars, Hashemi, Gerdeh, Hasani, and Gharib.

Interestingly, it is thought that origin of all the four cultivars were Guilan province, Iran. Gb and Hm have originated in a common region, whereas Hn and Gr originated in two distinct areas from each other and from two previously mentioned. It is assumed that the origin of Hm was Fuman and Soma'e-sara, Gb was Soma'e-sara and Fuman, Hn was Hashtpar, Talesh, Masal and Astarara, and lastly Gr was originated in Tarom. However, there is to our knowledge no support for this claim in the published scientific literature. The Alborz Mountain range forms a barrier between the supposed origins of Gr and the three other cultivars, in particular for Hn (Fig. 2). Geographical barriers can contribute to speciation (Darwin, 1985; Doebeli and Dieckmann, 2003). Interestingly, our findings support the presumed origins of the cultivars and geographical isolation effect on them.

Comparing recorded parameters of Gr and Hn, as two on the opposite sides, revealed that most parameters had a reasonable correlation. The parameters of NC, SC, and NVS showed a positive correlation; meanwhile they were negatively correlated with NSC, SCI, and RE. As discussed previously, there are some evidences that callus necrosis of rice resulted from ethylene through increase in cellular putrescine levels (Bajaj and Rajam, 1996). It was suggested that application of exogenous spermidine, which increases cellular spermidine levels and decreases cellular putrescine levels, adjusts putrescine/spermidine ratio (Bajaj and Rajam, 1996). Interestingly, it was indicated that indole-3-butyric acid (IBA) considerably enhances putrescine biosynthesis result in an increase of the putrescine/spermidine ratio; furthermore, auxin-induced root formation is thought either require or induce the promotion of polyamine (putrescine) synthesis (Friedman et al., 1985). There is increasing evidence that callus-browning trait is genetically controlled. However, either the root formation or callus necrosis obtained in the present investigation may be explained by considering all evidences, including the positive effect of ethylene on callus necrosis (Adkins et al., 1990; Songstad et al., 1991), ethylene-regulated putrescine/spermidine ratio, callus necrosis affected by putrescine/spermidine ratio (Bajaj and Rajam, 1996), casual relationship of ethylene and roots (Lorbiecke and Sauter, 1999), root formation through polyamine (putrescine) synthesis (Friedman et al., 1985), auxin-induced ethylene synthesis (Lorbiecke and Sauter, 1999).

However, the results of the present study by negative correlation between root formation and necrotic calli invitingly need more intense investigations.

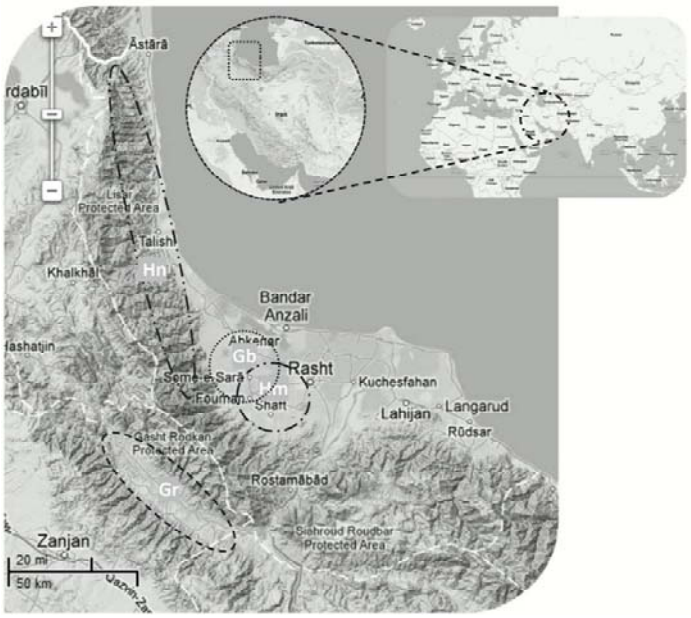

Figure 2: Origin of all the four cultivars was the province of Guilan, Iran (Google Maps 2012). Hasani (Hn), Gharib (Gb), Hashemi (Hm), and Gerdeh (Gr).

4 CONCLUSION

The results of this experiment proved that the most responsive and appropriate cultivar in callus initiation is in decreasing order: Gb > Hm >> Hn ≥ Gr. While Gb appeared very similar in the callogenesis and observed parameters to Hm, Hn and Gr represented dissimilarity among each other and the every other cultivar. The parameters of NC, SC, and NVS indicated a positive correlation; meanwhile they were negatively correlated with NSC, SCI, and at last RE. Gb and Hm produced actively, healthy and scutellar calli, therefore can be employed in tissue culture mediated breeding programs of Iranian Indica rice cultivars (Figure 3).

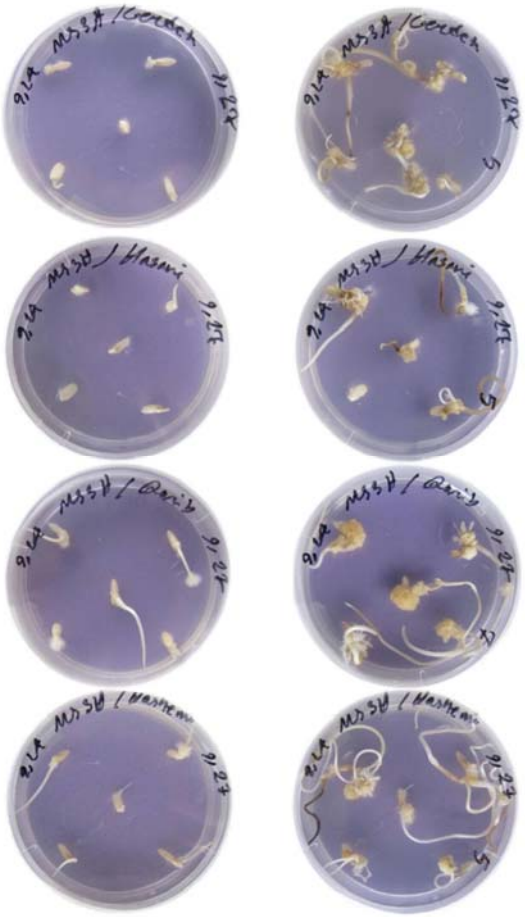

Figure 3: Callus growth from the four rice cultivars 4 days (left) and 24 days (right) after culturing on the callogenesis medium. Rice cultivars from up to down: Gerdeh (Gr), Hasani (Hn), Gharib (Gb), and Hashemi (Hm). Gr produced the lowest rate of scutellar calli (SC), while it had the highest rate of non-scutellar callus (NSC) and root emergence (RE). Although Hn produced the highest SC and the lowest NSC and RE, it showed the highest non-viable seeds (NVS), necrotic calli (NC), and slow growing callus (SGC). Gb produced the highest rate of appropriate callus (AC) because it had the highest SC and the second lowest NSC after Hn, furthermore with the lowest rate of NC and SGC in contrast to Hn. Hm produced high amount of AC but stood after Gb, since it had higher rate of NC, NSC, and above all grew four times slower than Gb.

5 ACKNOWLEDGMENT

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