Chapter 2

REVIEW OF LITERATURE
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Rust fungi are the largest group of plant parasitic fungi in Heterobasidiomycetes and cause severe diseases in many plants (Strange and Scott, 2005). The work on rust fungi is mainly on taxonomy with symptomatology and infection patterns. Some of these rusts are morphologically interesting as they show malformations due to infection. There are very few reports on biochemical and molecular studies on such rust fungi. However, the phytopathogenic fungi which can grow under artificial culture conditions have been studied for biochemical and molecular aspects of disease development. Variety of different techniques are also available for studying the process of sequential disease development. The available literature on different techniques as well as progress made in the study of disease development by fungal pathogens, in general and rust fungi in particular, is reviewed within the framework of this chapter.

International and National status of the work

The distribution of rusts is worldwide and the greatest number of species occur in temperate and near temperate regions and have been studied by many research workers (Narasimhan and Thirumalachar, 1961; Payak, 1957; Cummins and Hiratsuka, 2003; Manoharachary et al., 2005). Therefore the study of rust fungi has international importance.

Metabolic changes during disease development by fungal pathogens have been studied by many research workers, but reports specifically on rust fungi are very limited in number. Although the general metabolic changes incited by the rusts and other pathogenic fungi are similar, rust fungi in themselves stand apart in being obligate parasites causing minimum damage to the host. These metabolic changes can be analysed by biochemical study of host – pathogen
complex during disease development. The initiation of disease requires successful spore germination of the pathogen followed by entry inside the host plant. Tucker and Talbot (2001) have reviewed the mechanism of spore attachment and germination which is necessary for development of pre-penetration infection structures. The germination of the spores is highly dependent upon different physical factors like temperature and availability of humidity in the form of rain, dew or atmospheric moisture.

The role of moisture in the process of disease development has been well documented by Agrios (1997). Seem and Russo (1984) have demonstrated in case of cedar apple rust Gymnosporangium juniperi-virginianae, that the longer periods of leaf wetness are decisive in both the formation of basidiospores as well as the severity of infection. They reported that the severity of infection goes on increasing with longer leaf wetness period under optimum temperatures. Schmidt (2003) also demonstrated the importance of surface moisture in the form of rain or dew in infections of Cronartium quercuum and also stated that determining the optimum moisture requirement is important for the search of rust resistance. However, Bailey and Gurjar (1920) reported decrease in moisture content in the rust infected wheat plants. The occurrence of many diseases in different regions is dependent upon the rainfall within the year, e.g., Late blight of potato, downy mildew, apple scab are found in the areas with high rainfall or high relative humidity during the growing season (Agrios, 1997). Filajdic and Sutton (1992), Chiou (1997), Duveller et al. (1997), Gracia-Garza and Fravel (1998), Johnson and Sutton (2000), Becktell (2005), Liu and Xiao (2005), Rahaman and Punja (2005), Khandare et al. (2008) have also reported role of moisture in the disease development or increased moisture in various host – pathogen systems including lower as well as higher fungi.

Most of the fungi become independent of the external moisture levels after entry into the host tissues. The entry of the pathogen into the host initiates variety of different responses in the host. The most evident effect of fungal infection on the plants is appearance of lesions following infection (Agrios, 1997). The formation of lesions and the loss of chlorophyll pigments ultimately affect the photosynthetic machinery of the host (Carbonell et al., 1998). Shaw and Samborsky (1957) have reported decrease in the rate of
photosynthesis due to destruction of chlorophyll in wheat leaves infected by stem rust. Conceicao et al. (1997), Scarpari et al. (2005) have detected decrease in the chlorophyll due to infection by *Crinipellis perniciosa*, a basidiomycete, in cocoa. Kumar and Manoharachary (1985), Khan et al. (2001), Tavernier et al. (2007) have also shown the decrease in chlorophyll due to infection by various fungi. Livne et al. (1964) also reported decrease in the rate of photosynthesis in rust infected wheat and bean leaves. Along with changes in the chlorophyll contents, increase in the rate of respiration in rust infected plant tissues has been recorded by several research workers (Daly et al., 1957, 1962a, 1962b; Livne, 1964; Leonard and Szabo, 2005; Shaw and Samborsky, 1957). The same results have been recorded in other phytopathogenic fungal or bacterial systems by Bushnell and Allen (1962), Scott and Smillie, (1966), Fett et al. (1987), Nemec et al. (1989), Hatcher and Ayres, (1998), Gawande and Patil (2004). Daly et al. (1957) reported that even the pathways of respiration change during sporulation of the rust fungi in the host. But Bailey and Gurjar (1920) reported decrease in the rate of respiration in wheat plants infected with stem rust.

The establishment of compatible interaction in the rust – infected host massively increases the rate of respiration and alters the normal direction of phloem transport, to divert nutrients, mainly sugars, to the infected tissues (Salzman et al., 1998; Leonard and Szabo, 2005; Harrison, 1999; Voegele et al., 2001; 2006). Other host – pathogen systems also indicate alterations in the amount of reducing sugars due to pathogen infection (Chan and Thrower, 1980; Nemec et al., 1989; Conceicao et al., 1997; Khan et al., 2001; Chung and Tzeng, 2004; Gawande and Patil, 2004). Salzman et al. (1998) reported that the accumulation of sugars alongwith proteins constitutes a novel, developmentally regulated defense mechanism against phytopathogens in maturing grape fruit. They have also stated that accumulation of sugars is one of the physiological adaptation in plants that affect fungal pathogenesis, but it has received less attention in the studies. The changes in the metabolism of infected host is also reflected by changes in the levels of phenols, synthesis of proteins, free amino acids and individual amino acids like proline (Bajaj et al., 1983; Nicholson et al., 1986; Salisbury and Ross, 1986; Lagrimini, 1991; Issac, 1992; Liu et al., 1993; Osbourn, 1996; Chiou, 1997; Conceicao et al., 1997).
1997; Dharmadhikari and Jite, 1997; Hahn et al., 1997; Heath, 1997; Rey et al., 1998; Struck et al., 1998, 2004; Valette et al., 1998; Huckelhoven et al., 1999; Jite and Tressa, 1999; Gogoi et al., 2001; Khan et al., 2001; Lorena et al., 2001; Voegele et al., 2001, 2006; Wirsel et al., 2001; Cheong et al., 2002; Aghdaei et al., 2003; Gabler et al., 2003; Gachomo et al., 2003; Kiran et al., 2003; Kosack and Parker, 2003; Panstruga, 2003; Takenaka et al., 2003; Bakkeren and Gold, 2004; Balogun and Teraoka, 2004; Dagar et al., 2004; Katoch et al., 2004; Both et al., 2005; Chen and Dickman, 2005; Gabaldon et al., 2005; Guimil et al., 2005; Kishor et al., 2005; Pegard et al., 2005; Scarpari et al., 2005; Garcia – Brugger et al., 2006; Rana and Kaushal, 2006; Ranjini et al., 2006; Bhargava et al., 2007; Miranda et al., 2007; Muthukumar, 2007; Tavernier et al., 2007; Thakker et al., 2007; Upadhyaya and Gupta, 2007; Eichmann and Huckelhoven, 2008; Fung et al., 2008; Khandare et al., 2008; Satisha et al., 2008). Though the levels of proteins in different host – pathogen complex vary considerably, the change in the protein synthesizing activity in the infected tissues and surrounding the lesions can be attributed to the fungal synthesis of proteins (Katoch et al., 2004; Khandare et al., 2008).

The host plant responds to infection and growth of pathogen by raising defense responses. Glazebrook (2005) has reviewed different defense mechanisms in plants in response to infection by biotrophs and necrotrophs. Schulze – Lefert and Panstruga (2003) have extensively reviewed the strategies applied by biotrophic fungi who have stated that the biotrophic fungi suppress the activation of defense responses in immediate proximity of the infection site and also activate some host genes helping the fungus to establish the biotrophic relationship with the host. The enzymes like peroxidase and polyphenol oxidase are greatly induced in the host plants infected by variety of pathogens including rust fungi (Isaac, 1992; Agrios, 1997). Peroxidases are a group of diverse enzymes which are expressed in response to pathogen infection, symbiotic mycorrhizal infection, treatment with biocontrol agents, elicitation, wounding or stress and are believed to be involved in the resistance reactions (Stahmann et al., 1966; Seevers et al., 1971; Vance and Sherwood, 1976; Borchert, 1978; Lagrimini and Rothstein, 1987; Spanu and Bonfante – Fasolo, 1988; Southerton and Deverall, 1990; Lagrimini, 1991; Kerby and Somerville, 1992; Reimers et al., 1992; Mitchell et al., 1994; Ryals et al.,
1996; Agrios, 1997; Kiba et al., 1997; Sticher et al., 1997; Wojtaszek, 1997; Bestwick et al., 1998; Cipollini, 1998; Martinez et al., 1998; Valette et al., 1998; Vanacker et al., 1998; Huckelhoven et al., 1999; Jite and Tressa, 1999; Kristensen et al., 1999; Mittler et al., 1999; Yedidia et al., 1999; Grant and Loake, 2000; Kim et al., 2000; Pritsch et al., 2000; Reymond et al., 2000; Schenk et al., 2000; Vanacker et al., 2000; Gogoí et al., 2001; Khan et al., 2001; Percival, 2001; Song and Goodman, 2001; Vale et al., 2001; Bray, 2002; Cheong et al., 2002; García – Garrido and Ocampo, 2002; Kawaoka et al., 2003; Mould et al., 2003; Chen et al., 2004; Durrant and Dong, 2004; Hassni et al., 2004; Kamalakannan et al., 2004; Sedlarova et al., 2004; Guimil et al., 2005; Szalay et al., 2005; Zheng et al., 2005; Bindschedler et al., 2006; Breusegem and Dat, 2006; García – Brugger et al., 2006; Jakupovic et al., 2006; Torres et al., 2006; Zaninotto et al., 2006; Asselbergh et al., 2007; Choi et al., 2007; Mohamed et al., 2007; Mur et al., 2008; Fung et al., 2008; Goswami and Punja, 2008; Mandal et al., 2008; Silva et al., 2008). The role of peroxidases is also described in lignification, suberisation and in cell wall repair and thickening by Vance and Sherwood (1976), Mader and Amberg – Fisher (1982), Mader and Fussl (1982), Bajaj et al. (1983), Abeles and Biles (1991), Brisson et al. (1994), Dangl et al. (1996), Bernards et al. (1999), Lee et al. (2001), Odjakova and Hadjiivanova (2001), Serrano et al. (2004), Gabaldon et al. (2005) Lee et al. (2007). The cellular resistance responses like oxidative burst, hypersensitive response and programmed cell death are characterized by elevated levels of peroxidase (Kar and Mishra, 1976; Lagrimini and Rothstein, 1987; Abeles and Biles, 1991; Kiba et al., 1997; Wojtaszek, 1997; Martinez et al., 1998; Grant and Loake, 2000; Kawaoka et al., 2003; Sedlarova et al., 2004; Breusegem and Dat, 2006; Skopelitis et al., 2006; Torres et al., 2006; Zaninotto et al., 2006; Choi et al., 2007; Mur et al., 2008). Chen and Klessig (1991) and Durner and Klessig (1995) stated that the concentration of salicylic acid immediately around the pathogen induced lesions is quite high enough which can bind to peroxidase and catalase. Ryals et al. (1996) stated that such binding thereby suppresses the resistance reaction of the host and leading to successful lesion development. Bestwick et al. (1998) and Bernards et al. (1999) showed that peroxidases, purified as well as from host – pathogen complexes have multiple substrate specificity and
various pH optima. Several research workers have reported that purified horse raddish peroxidase (HRP) is involved in NADH oxidation and hydrogen peroxide generation which is linked to NADH oxidation (Akazawa and Conn. 1958; Yokota et al., 1965; Elstner and Heupel, 1976; Halliwell, 1978; Mader and Amberg – Fisher, 1982; Mader and Fussl, 1982; Miller et al., 1985). Along with peroxidases, polyphenol oxidases are also involved in oxidation of phenols to more toxic quinones. The quinones are more toxic to the infecting pathogen and may help in restricting the growth of the pathogen (Agrios, 1997). Polyphenol oxidases have a role in response to biotic and abiotic stress, in herbivory, disease resistance, wounding, lignification, regulating the photoreduction of molecular oxygen and plastidic oxygen levels along with their participation in phenylpropanoid pathway (Stahmann et al., 1966; Czech-Kozbowska and Krzywaanaski, 1984; Constabel et al., 1995; Agrios, 1997; Jite and Tressa, 1999; Khan et al., 2001; Orozco – Cardenas et al., 2001; Balogun and Teraoka, 2004; Kamalakannan et al., 2004; Vallad and Goodman, 2004; Hu – zhe et al., 2005; Szalay et al., 2005; Walters et al., 2006; Lee et al., 2007; Miranda et al., 2007; Thipyapong et al., 2007; Silva et al., 2008). The reports of Jite and Tressa (1999), Walters et al. (2006), Miranda et al. (2007). Silva et al. (2008) indicate the role of polyphenol oxidase during the infection of rust fungi to their respective hosts, but majority of other reports are in lower fungi or in response to abiotic stresses.

The infecting pathogen gains entry into the host through mechanical or enzymatic means by degrading the cell wall (Howard et al., 1991; Knogge, 1996; Xu and Mendgen, 1997; Latunde – Dada, 2001; Bakkeren and Gold, 2004; Both et al., 2005). The enzymatic degradation of cell wall involves expression of different cellulases and xylanases and the invasive ability of the infecting pathogens are dependent on its ability to produce cellulase (Tong et al., 1980; Sazci et al., 1986; Fett et al., 1987; Cervone et al., 1989; Isaac, 1992; Heiler et al., 1993; Alghisi and Favaron, 1995; Knogge, 1996; Agrios, 1997; Eriksen and Goksoyr, 1977; Xu and Mendgen, 1997; Rey et al., 1998; Salzman et al., 1998; Punnapayak et al., 1999; Yedidia et al., 1999; Eshel et al., 2000; Staples, 2000; Downer et al., 2001a, 2001b; Latunde – Dada, 2001; Taylor and Whitelaw, 2001; Gabler et al., 2003; Lev and Horwitz, 2003; Li et al., 2003; Mould et al., 2003; Bakkeren and Gold, 2004; Maheshwari, 2005;
Khalid et al., 2006; Asoufi et al., 2007; Okafor et al., 2007; Eichmann and Huckelhoven, 2008). But the reports of Lev and Horwitz (2003) and Li et al. (2003) indicate that the cellulases are not involved in pathogenesis by some of the fungi. The role of extracellular enzymes, in general and cellulases in particular, is therefore reported to be uncertain in disease development (Bakkeren and Gold, 2004).

With advancement of the disease, the metabolic balance of the host continues to collapse and the infected host may show some drastic changes in general physiology and metabolism. The normal growth and development of a plant depends upon critical regulation of phytohormone balance (Bryant and Lane, 1979; Woodward and Bartel, 2005; Zhao, 2008). The infection of a plant by pathogen frequently leads to malformations in the host which is taken as an indication of overproduction of phytohormones (Isaac, 1992; Agrios, 1997; Chung and Tzeng, 2004). Production of malformations in the infected plant tissues may be due to different growth regulators. One of the important phytohormone from the plants is indole 3 – acetic acid (IAA) which controls different processes and growth responses in plants (Hinsvark et al., 1954; Bryant and Lane, 1979; Bialek et. al., 1992; Abel and Theologis, 1996; Ballester et al., 1999; Lobello et al., 2000; Agrios, 1997).

Several fungal and bacterial plant diseases are known to cause hyperauxiny and in most of such cases, the infecting organism produces IAA under culture conditions (Wolf, 1952; Epstein and Miles, 1967; Chan and Thrower, 1980; Fett et al., 1987; Frankenberger and Poth 1987; Isaac, 1992; Agrios, 1997; Robinson et al., 1998; Grsic-Rausch et al., 2000; Gardan et al., 1992; Beattie and Lindow, 1999; Li et al., 2003; Chung and Tzeng, 2004; Devos et al., 2005; Lahey et al., 2004; Manzhalesava et al., 2004; Maor et al., 2004; Leveau and Lindow, 2005; Mendez – Moranel et al., 2005; Gravel et al., 2007). The crown galls and hairy roots induced in the plants due to Agrobacterium sp. involve the overproduction of auxins and cytokinins in the infected plants (Isaac, 1992; Agrios, 1997). But Tudzynski (1997) reported several reasons for treating correlation between overproduction of IAA and gall induction with caution. Recent report by Reineke et al. (2008) shows that hyperauxiny is also caused in the host plants due to infection with mutant strains of Ustilago maydis unable to produce IAA. The increased source of IAA in the infected
plants may be due to increased synthesis of IAA or due to decreased activity of IAA degrading enzyme – IAA oxidase (IAAO) (Hangarter and Good, 1981; Woodward and Bartel, 2005). Wiese and DeVay (1970), Lee (1971), Bryant and Lane (1979), Mills and Todd (1973), Bajaj et al. (1983) have reported that activities of IAAO are affected by biotic and abiotic stresses and also by the levels of IAA. Jite and Tressa (1999) and Chung and Tzeng (2004) attributed the increased IAA content in the malformed tissues of the host to the decreased IAAO activity. But contents of IAA and IAAO activities could not always be correlated (Manzhalesava et al., 2004). The role of IAA in plant disease development still remains speculative.

Gibberellic acid is one of the other growth regulators whose overproduction is reported in some of the plant disease. Gibberellin metabolism and its regulation is reviewed by Yamaguchi (2008). Production of various gibberellic acids is reported in different bacteria and fungi, most of which are lower fungi (Bailiss and Wilson, 1967; Zeigler et al., 1980; Matheussen et al., 1991; Kenmoku et al., 2001; Hasan, 2002; Malonek et al., 2005; Karakoc and Aksoz, 2006; Kawaide, 2006). Currently 126 different types of GAs are known (MacMillan, 2002) and there are very few fungi (Gibberella fujikuroi, five species of Sphaceloma and Neurospora crassa) which produce only a limited number of GAs known till date (Kawaide, 2006). Out of these fungi, only G. fujikuroi and Sphaceloma sp. (eg. Sphaceloma manihoticola) are phytopathogenic and cause super-elongation disease (Tudzynski, 1997; Zeigler et al., 1980). GAs are also implicated to have protective roles in plants against diseases (Smith, 1998; Eshel et al., 2000; Santos et al., 2000). Schwechheimer (2008) has reviewed the mechanisms involved in GA signalling but the role of GA in disease development by fungi is not fully understood.

The biochemical analysis of the host – pathogen complex can be utilized to get insights into detail molecular analysis of the process of disease development. There are numerous reports of cDNA analysis of individual genes or complete transcriptome of the organisms subjected to abiotic as well as biotic stress including pathogen infection (Hahn et al., 1997; Hahn and Mendgen, 1997; Harrison, 1999; Dufresne et al., 2000; Turner et al., 2002; Dodds et al., 2004; Warren and Covert, 2004; Kemen et al., 2005; Kishor et al., 2005; Khurana et al., 2005; Catanzariti et al., 2006).
The study of host–pathogen complex relies mainly upon the identification of the pathogen. Traditional approaches to identify fungal pathogens, in general and rusts, in particular involve heavy use of morphological characters (Rezende and Dianese; 2001; Bermdt, 2002; Cummins and Hiratsuka, 2003). Relative paucity of morphological characters often results in lack of agreement on the phylogenetic or taxonomic status of the fungi (Wingfield et al., 2004). Swan and Taylor (1993) and Vogler and Bruns (1998) have discussed the advantages of DNA data for molecular and phylogenetic analysis. Traditional identification and/or phylogenetic approaches hence need to be complemented with the DNA sequence data which can be used for phylogenetic studies (Hamer et al., 1989; Bruns et al., 1990; Kretzer et al., 1996; Roy et al., 1998; Kurkela et al., 1999; Borneman and Hartin, 2000; Beszteri et al., 2001; Nascimento and Rossi, 2001; Wu et al., 2002, 2003; Anikster et al., 2004; Ramsfield and Vogler, 2004; Pei and McCracken, 2004; Aime, 2006; Scholer and Aime, 2006; Merwe et al., 2007). The molecular data based on DNA or protein sequence information can be efficiently utilized for constructing the phylogenies of the desired group of organism with the help of different softwares. The routine method of phylogenetic studies involves the basic local alignment search tool (BLAST) analysis for similarity searches. BLAST search finds the regions of statistically significant local similarity in DNA or protein sequences with the help of databases. BLAST search can be used for identifying the functional or evolutionary relationships between sequences and identifies the members of the related gene families (Zhang et al., 2000). The alignment of the sequences retrieved from the BLAST analysis can be done with various computer assisted methods using softwares like Clustal W (Thompson et al., 1994). The aligned dataset may then be subjected to phylogenetic analysis with variety of different softwares. These softwares make use of different algorithms for phylogenetic tree building based on various phylogenetic assumptions. These are based on similarity or divergences in the sequences to be compared (Saitou and Nei, 1987; Saitou and Imanishi, 1989; Bulmer, 1991; Rzhetsky and Nei, 1993; Ren et al., 1995; Kumar, 1996; Bryant and Waddell, 1998; Steel and Penny, 2000; Takahashi and Nei, 2000; Gascuel, 2000. Gascuel et al., 2001; Desper and Gascuel, 2002; Pupko et al., 2002; Kumar et al., 2004; Silva et al., 2005; Levy et al., 2006).
The phylogenetic study based on DNA or protein sequences has several advantages over the analysis using morphological characters. The molecular data provides few thousand characters to be analysed at a single time and these characters are independent of the host or pathogen. Several reports on such analyses in case of fungi indicate the agreement with previous taxonomic treatments or suggest modification in the present taxonomic status of the genus based on its phylogenetic affinities (Hamer et al., 1989; Roy et al., 1998; Ramsfield and Vogler, 2004; Wingfield et al., 2004; Aime, 2006; Scholler and Aime, 2006).

Studies of rust fungi are mainly hampered due to their obligate parasitic nature which requires a living host to complete the life cycle. Most of the knowledge of disease development by biotrophs stems from the studies on non obligate biotrophs. The rust fungi themselves are interesting due to their obligate parasitic nature causing minimum damage to the host plant in spite of establishment of the intimate relationship with the host. The studies on the processes that determine the fungal development and cause disease, especially in case of obligate parasites, require highly sophisticated technologies like simultaneous high-throughput analysis of large number of genes using microarray technology (Both et al., 2005). The other alternative to expensive technologies is successful in vitro culture of these fungi which will allow various growth phases of obligate parasites to be analysed in detail. But the successful in vitro culturing in case of rust fungi remains a difficult task.

Hotson and Cutter (1951) successfully demonstrated the in vitro cultures of *Gymnosporangium juniperi – virginianae* which had the potential to reinfect the hosts and produce characteristic symptoms. Allen et al. (1988), Hu and Amerson (1991), Diner (1999), Moricca et al. (2000), Pie and Gibbs (1992), Allen et al. (1988), Hu and Amerson (1991) also reported culturing of various rust fungi in vitro. But the in vitro growing rust fungi remain a slow growing organisms and requirements for their growth and development remain largely unknown. The recent study of in vitro differentiation of infection structures in *Puccinia graminis* f. sp. *tritici* by Wiethölter et al. (2003) indicated that the in vitro differentiation rates of late infection structures like haustorial mother cells and haustoria are very slow. But a combination of physical and chemical signals induced the in vitro differentiation of haustorial mother cells which
were cytologically identical to those formed in planta. They also concluded that the efficient in vitro differentiation of haustorial mother cells will allow the analysis of molecular details of the processes involved in the induction and differentiation of this critically important developmental stage of the economically important plant pathogenic rust fungi. Hence, the in vitro cultures of rust fungi have exceedingly important advantage in studies of disease development by rust fungi. Maheshwari et al. (1967) have attempted to establish the rust fungi in the callus tissues of the host. Though they reported the proliferation of the host tissue, the rust mycelium did not invade the newly formed tissues. The associated cultures of rust fungi in the host callus may also serve some important purposes of the study.

The review of literature thus revealed that there are drastic changes in the host due to phytopathogenic organisms. The biochemical studies in disease development by rust fungi in particular were found to be very scanty. Majority of the international reports indicate the studies at molecular level and large amounts of significant data has been collected in Uromyces sp. infecting variety of important crop plants. The biochemical analysis of disease development by rust fungi inducing malformations in the host has not been reported yet at national level. Such rust fungi provide advantage of morphological monitoring of the disease progression. In the present studies, two host – pathogen systems (Acacia eburnea – Ravenelia esculenta and Jasminum officinale var. grandiflorum – Uromyces hobsoni) were morphologically reviewed with the help of reports by Narasimhan and Thirumalachar (1961) and Payak (1958). These rust fungi, inducing malformations in the host, were biochemically analyzed during progressive stages of disease development. Analysis was done with procedures from the established protocols. However few protocols were modified and are described in the section of ‘Materials and Methods’.